PERSISTENT ORGANIC POLLUTANTS

IN MARINE BIOTA:

ENVIRONMENTAL AND HUMAN HEALTH RISKS

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K *starts the Knowledge and ends the darK,*

A *is in the nAture and in humAnity,*

Y *is in You and everYdaY.*

I dedicate all the letters ‘K’, ‘A’ and ‘Y’ in this thesis to my wife.

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SUMMARY

In 2001, 122 nations (including Singapore) signed the Stockholm Convention (UNEP) to phase out a suite of 12 persistent organic pollutants (POPs) considered as a potential risk to the environment and human health. The objective of this research was to investigate the occurrence of POPs in Singapore's marine biota, the bioaccumulation mechanisms in aquaculture fish and shellfish, and ultimately the exposure of Singapore's population through the consumption of seafood. POPs of interest included the polychlorinated biphenyls (PCBs), organochlorine pesticides, and the polybrominated diphenyl ethers (PBDEs). Detailed laboratory and field studies have been undertaken on the measurement, distribution and fate of POPs in Singapore's marine biota and seafood, coupled with human risk assessment studies on seafood processing and consumption.

A fast, sensitive and robust analytical method, using microwave assisted extraction (MAE), was optimized and validated for the analysis of POPs, including PBDEs, in marine biota tissues. The choice of MAE has resulted in important savings in terms of solvent consumption and extraction time and enabled the analysis of a large number of samples for this project.

The green mussel, *Perna viridis,* was used in this study as an effective bioindicator for PCBs, pesticides and, for the first time, PBDEs in the marine environment. The geographical distribution of POPs in green mussel samples revealed the ubiquity of these POPs in the coastal waters of Singapore and the presence of "hot spots" of contamination

associated with industrial and shipping activities. Mussel tissue extracts were then screened for hormone activities using a human-cell based reporter gene bioassay. Significant correlations exist between androgenic activities, in the presence of dihydrotestosterone, and the total concentration of POPs, offering new understanding of the presence and potential impacts of endocrine disrupting chemicals on marine biota.

POPs were quantified in 24 mangrove species collected at two sites in Singapore. A biomagnification phenomenon was observed amongst the mangrove species. PBDEs and Chlordane congener profiles varied amongst species. No clear difference was observed between the two sites located on each side of the Straits of Johore. Comparison with other studies suggests potential for ecotoxicological impacts on organisms at higher trophic levels in the mangrove food web, including mammals and birds.

The comparative growth rates and POPs bioaccumulation were monitored in the Pacific Oyster, *Crassostrea gigas*, at two sites in Singapore, one 'clean' and one 'contaminated'. Results show that marine pollution represents a specific threat to both the yield and quality of oyster tissues, and therefore to the oyster aquaculture industry in Singapore. On a positive note, the effects of pollution on oyster growth rates and contaminant burden were found to be reversible.

The ingestion exposure of Asian seabass to *p,p'*-DDT were evaluated in a simulated aquaculture system. The bioaccumulation mechanims (uptake efficiency, metabolism, and tissue partitioning) following ingestion exposure to *p,p'*-DDT at environmentally relevant

levels (ng/g range in fish meal) were different from the unrealistic dosage levels used in previous environmental modeling studies. Data underline the importance of fish meal quality on the aquaculture final product, and therefore on human food safety.

POPs levels were measured in the edible portions of 20 different seafood types consumed in Singapore. Chlordane, PCBs, DDT were the main POPs found amongst the seafood types, with highest concentrations in salmon fillets and green mussels. Daily intakes of POPs from seafood are below the oral reference dose set by the US FDA. Daily intake of DDTs, Heptachlor and PCBs in seafood exceeded the conservative cancer benchmark concentrations set by the US EPA, suggesting that a significant number of people are potentially at risk in Singapore over a lifetime from seafood consumption.

Cooking of raw fish contaminated with POPs is expected to reduce the consumption exposure risk to human health. Cooking effects decreased the load of POPs in salmon steak with an average loss of 26±15% relative to the initial POP load in the raw steak, with no significant differences between cooking methods. The removal of the skin from the cooked salmon steak resulted in a further average loss of 9±3%.

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NOMENCLATURE

Symbols

K_{oa}	Octanol-air partition coefficient
K_{ow}	Octanol-water partition coefficient
m/z	Mass to charge ratio
p	Significance level of correlation
r	Pearson coefficient of linearity

Abbreviations

AR	Androgen Receptor
ASE	Accelerated Soxhlet Extraction
BDE	Brominated Diphenyl Ether
BLD	Below Limit of Detection
CB	Chlorinated Biphenyl
CHL	Chlordane
DCM	Dichloromethane
DDD	1,1-dichloro-bis(p-chlorophenyl)ethane
DDE	1,1-dichloro-bis(p -chlorophenyl)ethylene
DDT	bis(p-chlorophenyl)-1,1,1-trichloroethane
DHT	Dihydrotestosterone
DL	Detection Limit
dw	dry weight

E2	17β-estradiol
EDC	Endocrine Disrupting Chemical
EI	Electron Impact
EPA	Environmental Protection Agency
ER	Estrogen Receptor
EROD	Ethoxyresorufin-*O*-de-ethylase
FDA	Food and Drug Administration
GC-MS	Gas Chromatography – Mass Spectrometry
GPC	Gel Permeation Chromatography
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HD	High Dose
ICP-MS	Induced Coupled Plasma – Mass Spectrometry
IUPAC	International Union of Pure and Applied Chemistry
LD	Low Dose
LRT	Long Range Transportation
lw	lipid weight
MAE	Microwave Assisted Extraction
MDI	Mean Daily Intake
MDL	Method Detection Limit
MRL	Maximum Residue Limit
MW	Molecular Weight
NIST	National Institute of Standards and Technology

OCP	Organochlorine Pesticide
PBDE	Polybrominated Diphenyl Ether
PCA	Principal Component Analysis
PCB	Polychlorinated Biphenyl
PCNB	Pentachloronitrobenzene
PTFE	Polytetrafluoroethylene
RfD	Reference Dose
RSD	Relative Standard Deviation
RSYC	Republic of Singapore Yacht Club
SD	Standard Deviation
SFE	Supercritical Fluid Extraction
SIM	Selected Ion Monitoring
SRM	Standard Reference Material
STEL	Short Term Exposure Limit
TBT	Tributyl Tin
TMSI	Tropical Marine Science Institute
TVL	Threshold Value Limit
UNEP	United Nations Environmental Program
ww	wet weight

CHAPTER I – INTRODUCTION

I – 1 Background

Persistent organic pollutants (POPs) are pollutants of major concern that have potential impacts upon the environment and human health. Due to their propensity for transboundary transportation, POPs are a regional as well a global environmental issue (Jones et de Voogt, 1999). Environmental POPs have been studied quite intensively since 1970's, when the harmful effects of DDT and PCBs on wildlife species were first detected. Nowadays, major international institutions, such as the United Nation Environmental Program (UNEP), the Food and Agriculture Organization of the United Nations, and the World Health Organization, have established programmes to investigate the behavior of POPs in the global environment. However, an imbalance is apparent in the understanding of POPs amongst the different regions of the world. In America and Western Europe, governments are actively monitoring POPs in the environment (e.g. Greenfield et al., 2003; Kemmlein et al., 2003) and advanced environmental models at both the global and molecular scales (Jones et de Voogt, 1999). In contrast, there is almost no existing data in Sub-Saharan Africa, Central America and the Caribbean, the Indian Ocean and much of Asia (UNEP, 2003). Asia has been identified as major source of POPs at the global scale (Iwata et al., 1993), and current voids in the environmental database for Asia seriously impairs the understanding of the global fate and transfer of POPs. The importance of tropical regions in the global distribution of POPs has also been highlighted in the 'global distillation' theory, whereby POPs are volatilized in tropical and temperate regions to eventually condense at the poles (Bard, 1999). However,

once again, the occurrence of POPs at the tropics has been poorly documented (Miao et al., 2000).

In 2001, 122 nations (including Singapore) signed the Stockholm Convention under the UNEP to phase out a particular suite of POPs considered as priority contaminants (UNEP, 2001). The "red list" of contaminants includes nine organochlorine pesticides, polychlorinated biphenyls (PCBs), dioxins and furans. Signatories of the Stockholm Convention agreed to ban the production, import and use of red list POPs. Within two years from the ratification, signatories undertake to perform an inventory of the sources and discharges of POPs into the environment, to set up adequate regulations and promote environmental education and awareness in this field (UNEP, 2001).

On top of these red list POPs, the Stockholm Convention invites signatories to identify other POPs of potential concern. In particular, the polybrominated diphenyl ethers (PBDEs), used as flame retardants, have emerged in the last decade as potential 'new' POPs of environmental concern. PBDEs have structural similarities to PCBs, and are suspected to have similar environmental and toxicological characteristics. Most strikingly, PBDEs have been found in all environmental compartments, particularly in air, marine sediments, human breast milk and polar species tissues, revealing the widespread nature of PBDE contamination (De Wit, 2002). Although environmental levels of PBDEs can be traced back to the 1970's in Europe and America (De Wit, 2002), there is no existing data in South-east Asia. To date, only the European Union has taken measures to control the use of PBDEs, and

has recently banned the production and use of pentabrominated formulations (Kemmlein et al., 2003).

POPs migrate to the marine environment via unintentional human release, river discharge or atmospheric deposition (Vallack et al., 1998). POPs have been detected in all types of water bodies, from the deep-sea to surface sea water, from rivers to mountain lakes. Due to their lipophilic nature, POPs accumulate in marine food chains to eventually reach peak concentrations in top predators. POPs have a wide range of toxicological effects and their bioaccumulation has resulted in widespread environmental impacts. Some POPs, such as aldrin or endrin have a very high acute toxicity to aquatic organisms (UNEP, 2002b), but other effects can be more insidious. For example, POPs are implicated in the impaired breeding success of fish-eating birds (Connell et al., 2003). In the Arctic, immunological and sexual disorders have been observed in polar bears, as a consequence of the level of POPs in their tissues (Bard et al., 1999).

POPs readily accumulate in human tissues and represent a threat to human health. A wide range of toxicological effects on humans are likely, including carcinogenicity and endocrine disruption. POPs accumulate in lipid-rich tissues, particularly in the breast milk of nursing mothers, representing the greatest risks for infants. Studies in America, Europe and Japan have revealed that ingestion of POPs from food, and particularly seafood, is a major route of exposure to adults (Bocio et al., 2003, Greenfield et al., 2003; Smith and Gandolli, 2002). As a result, concentrations of POPs in lactating mother's milk were positively correlated with seafood consumption in Japan (Ohta et al., 2002). Most controversially, in the USA, it has

been shown that children born to women who had consumed contaminated fish from the Great Lakes had a reduced IQ and reading capacity (Jacobson and Jacobson, 1996).

Due to the increasing global human population and widespread depletion of natural fish stocks, the world is placing increasingly emphasis on aquaculture to meet food needs. However, recent scientific reports on contamination of salmon tissue with a range of POPs have highlighted the human health risks associated with the consumption of aquaculture products contaminated with POPs (Antunes and Gil, 2004; Hites et al., 2004). Such studies have generated a vociferous debate, and created an immediate negative impact on the farmed salmon aquaculture business. Although scientific studies were conducted in laboratory conditions to evaluate the uptake of pollutants, there is very limited information on the accumulation of POPs in aquaculture systems. In 2003, total aquaculture production in Singapore totaled S$104 million in 2001 (http://www.ava.gov.sg). There remains considerable growth potential for development of Singapore's aquaculture industry, but this aspiration must be balanced with worldwide concerns over food security and product quality. There is a justifiable need for the enhanced monitoring and understanding of mechanisms of POPs in tropical aquaculture systems, such as Singapore.

I – 2 Study Objectives

This research focuses on the occurrence of POPs in Singapore's marine biota, their bioaccumulation in aquaculture fish and shellfish, and ultimately the exposure of POPs to the

human population of Singapore via the consumption of seafood. In particular, the specific objectives of the research, and its scope, are as follows:

1. To develop a robust, sensitive, rapid and quality assured analytical method for the detection of POPs, including PBDEs, in a large number of marine biota tissues. Refer to Chapter IV.
2. To assess the extent of POP pollution Singapore's marine environment. More specifically: (a) to identify key POPs in Singapore's marine environment using the green mussel, *Perna viridis*, as a bioindicator organism; (b) to quantify POPs using this bioindicator and place Singapore into an international context; (c) to evaluate the geographical distribution of POPs in Singapore's marine environment and identify potential contamination sources; and (d) to investigate the relationship between POPs and endocrine activity in Singapore's marine environment. Refer to Chapter V.
3. To investigate the occurrence of POPs in the mangrove ecosystem of Singapore. More specifically: (a) to assess biomagnification of POPs in mangrove food webs; (b) to compare metabolism of POPs amongst organisms; (c) to compare sites with different prevailing contamination levels; and (d) to evaluate the risks of POPs exposure for organisms at high trophic levels in the food chain. Refer to Chapter VI.
4. To assess the impact of POPs on the aquaculture of the Pacific oyster, *Crassostrea gigas*. More specifically: (a) to study the accumulation of POPs in oysters over an aquaculture cycle; (b) to compare growth and pollutant load at two sites with different prevailing contamination levels; and (c) to examine the

reversibility of adverse exposure to pollution, and particularly the depuration of POPs from oyster tissues. Refer to Chapter VII.

5. To investigate the dietary exposure mechanisms of the seabass, *Lates calcarifer*, to DDT pesticide in a controlled aquaculture experiment at environmentally relevant exposure levels. More specifically: (a) to evaluate the partitioning of DDT in the fish exposed to different doses, with an emphasis on the edible portions; (b) to characterize the uptake and metabolism processes of DDT in aquacultured seabass; and (c) to assess the risks associated with the presence of the pesticide in the aquaculture system. Refer to Chapter VIII.
6. To evaluate the risks associated via the consumption of POPs in seafood by the human population of Singapore. More specifically: (a) to measure the levels of POPs in seafood commonly consumed in Singapore, (b) to quantify the mean daily intake of contaminants for the general population in Singapore, (c) to compare levels with legal maximum residue limits; and (d) to use certified tools to determine carcinogenic and non-carcinogenic risks associated with the consumption of seafood in Singapore. Refer to Chapter IX.
7. To evaluate the effect of cooking on the intake of POPs from farmed salmon. More specifically: (a) evaluate the losses of POPs during baking, microwave cooking, boiling and pan-frying of salmon steak; (b) evaluate the losses of POPs as a result of skin removal; (c) understand the role of lipids on the loss of POPs from salmon tissues; and (d) to re-evaluate the risks associated with the consumption of salmon after cooking . Refer to Chapter X.

II – LITERATURE BACKGROUND

II – 1 Presentation of persistent organic pollutants (POPs)

II – 1 – 1 Definition

Organic chemicals that are lipophilic, persistent, toxic to both fauna and humans, and have the potential to undergo long range transportation are known as persistent organic pollutants or POPs (Vallack et al., 1998). The 'red' list of POPs defined by the UNEP (2001) includes: aldrin, dieldrin, endrin, DDT, chlordane, heptachlor, hexachlorobenzene (HCB), toxaphene, mirex, polychlorinated biphenyls (PCBs), dioxins and furans. In addition, other anthropogenic contaminants, such as polybrominated diphenyl ethers (PBDEs), polychlorinated naphtalenes and chlorinated paraffins, have attracted attention from the scientific community as they are regarded as "new classes" of POPs (Alcock and Jones, 1999). Hexachlocyclohexanes (HCHs) are persistent toxic substances, but are less bioaccumulative than organochlorine pesticides. Due to their environmental concern, HCHs are generally defined together with POPs by UNEP.

II – 1 – 2 Chemical structures and nomenclature

The chemical formulae of some common POPs are presented in Figure II-1. The nomenclature for the various isomers of chlordanes and HCHs utilizes Greek symbols, i.e. α- (or cis-) and γ- (or trans-) for chlordanes; and α-, β-, γ- and δ- for HCHs. PCBs are a family of 209 congeners, with a degree of substitution ranging from 1 to 10 chlorine atoms on the

biphenyl structure, identified according to the nomenclature by the International Union of Pure and Applied Chemistry (IUPAC). PBDEs are commonly named according to IUPAC nomenclature for PCBs with similar levels of substitution (De Wit, 2002). The level of substitution has a major influence on physical properties, such as lipophilicity, volatility, water solubility and biodegradability (Miao et al., 2000). General physical properties of POPs are detailed in literature reviews (e.g. Palm et al., 2002; De Wit, 2002) and in the UNEP database (http://www.chem.unep.ch/pops).

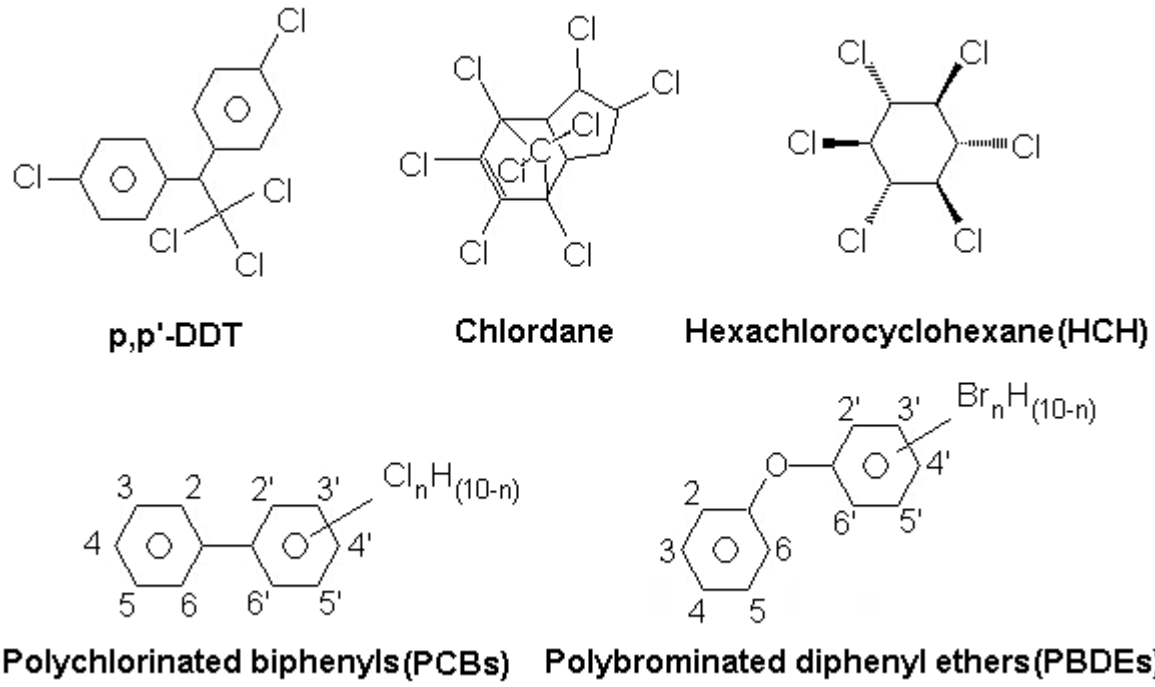

Figure II-1: Chemical structures of major POPs. Numbers for PCBs and PBDEs refers to the halogen substitution position as standardized by the IUPAC.

II – 1 – 3 Lipophilicity

POPs are characterized by a low water solubility, which is illustrated by a relatively high octanol-water partition coefficient (K_{ow}) (See Table II-1). As a consequence, POPs partition

strongly in aquatic systems and soils to solid and organic phases to avoid the aqueous phase (Jones and de Voogt, 1999). Eventually, hydrophobicity drives POPs to accumulate in fatty tissues of organisms and to biomagnify in food chains.

POP	MW (g/mol)	K_{ow}	References
p,p'-DDT	354.5	6.0	Gobas et al., 1988
p,p'-DDD	320.1	5.5	www.chem.unep.ch
p,p'-DDE	318.0	5.7	www.chem.unep.ch
chlordanes	409.8	6.0	Gobas et al., 1988
heptachlor	373.3	4.4-5.5	www.chem.unep.ch
HCH	290.9	3.8	www.chem.unep.ch
mirex	545.6	7.5	Gobas et al., 1988
tri-CBs	257.5	5.5-5.9	www.chem.unep.ch
tetra-CBs	292.0	5.6-6.5	www.chem.unep.ch
penta-CBs	326.5	6.2-6.5	www.chem.unep.ch
hexa-CBs	361.0	6.7-7.3	www.chem.unep.ch
hepta-CBs	395.5	6.7-7	www.chem.unep.ch
octa-CBs	430.0	7.1	www.chem.unep.ch
nona-CBs	464.5	7.2-8.2	www.chem.unep.ch
deca-CB	499.0	8.3	www.chem.unep.ch
tetra-BDEs	485.8	5.87-6.16	Rahman et al., 2001; Palm et al., 2002
penta-BDEs	564.7	6.64-6.97	Rahman et al., 2001; Palm et al., 2002

Table II-1: Molar weight and octanol-water coefficient (K_{ow}) of major POP contaminants.

Biomagnification is not expected for contaminants with a K_{ow} lower than 5 and will be significant for a K_{ow}>6.3 (van der Oost et al., 2003). HCHs have a K_{ow} of 3.8 and are therefore less bioaccumulative than PCBs and organochlorine pesticides.

II – 1 – 4 Persistence

POPs can resist chemical degradation via oxidation, reduction, hydrolysis, photolysis, free radicals, and other environmental chemical reactions (Bard, 1999), and are also recalcitrant to biological degradation (Vallack et al., 1998). As POPs are chemically stable, a long half-life in the various environmental media can be expected. As an example, the half-life of chlordane is estimated to be up to 14 years in soils (Hunter et al., 1995).

II – 1 – 5 Long-range transportation

The volatility/solubility properties of POPs, combined with their resistance to degradation, make possible the long-range transportation (LRT) of these contaminants at both the regional and global scales (Albaiges, 2003). Non volatile and highly hydrophobic compounds (e.g. heavy PBDEs, mirex) undergo LRT through adsorption on suspended solids in air and water media. Some other POPs, such as HCB, partition more readily between air and condensed phases (soil, water, vegetation) and undergo LRT through cycles of evaporation and re-deposition. Then, more water soluble compounds, such as HCHs, can undergo LRT dissolved in aqueous phases, e.g. rain, river, oceanic transportation (Albaiges, 2003). At low temperatures, deposition of POPs become dominant compared to the volatilization process. As a result, POPs are, at a global scale, volatilized at mid- and low-latitudes and are

eventually condensed and precipitated at the poles. This phenomenon is known as the global distillation theory (Bard, 1999).

II – 1 – 6 Usage of POPs

POPs have been used in a wide range of applications, as described in Table II-2. PCBs, PBDEs and chlordane were used as complex technical mixtures of many chemical congeners or compounds, whereas DDTs and HCHs comprised more simple technical mixtures (Bard, 1999).

POP	Pesticide	Plasticizer	Flame retardant	Dielectric fluid	Combustion/industrial by-products
aldrin	+				
chlordane	+				
DDT	+				
HCB	+				+
HCHs	+				
heptachlor	+				
mirex	+	+	+		
PCBs		+		+	
PBDEs			+		
dioxins					+
furans					+

Table II-2: Examples of usage of major POPs (adapted from Vallack et al., 1998).

PBDEs are widely used as flame retardants in a range of construction materials, textiles and other consumer products, such as electronics. In particular, penta-BDEs are incorporated in

polymers such as phenolic resins, polyvinylchloride, polyurethane, unsaturated polyesters, rubber, paints and textiles (Rahman et al., 2001). The carbon-bromine bond in PBDEs is weak, allowing their decomposition at temperature of about 50°C below the host material and preventing the formation of flammable gas when heated (Rahman et al., 2001).

II – 2 Analysis of POPs in biological tissues

II – 2 – 1 Introduction

POPs are generally present in the ng/g range in biological tissues (e.g. De Wit, 2002). Biological samples represent complex matrices, in which moisture, lipids and other organic molecules are potential sources of interference in the isolation, identification and quantification of POPs (Jayaraman et al., 2001; Strandberg et al., 1998). As a result, a large part of analytical organic chemistry has been dedicated, in the last 40 years, to the development and optimization of methods for the extraction of POPs, the removal of analytical interferences and their effective quantification. The most common steps for POPs analysis in biological tissues include solvent extraction, extract cleanup and quantification following gas-chromatography (GC).

II – 2 – 2 Solvent extraction of POPs from biological tissue

Current published methods for the extraction of PCBs and organochlorine pesticides from marine biological tissues include Soxhlet extraction (Wu et al., 2001), column elution (Falandysz et al., 2001) and manual shaking/centrifugation procedures (Stefanelli et al., 2004). These methods are rather time and solvent consuming. An alternative, supercritical

fluid extraction (SFE) (Miao et al., 2000), reduces analytical time and avoids use of hazardous organic solvent, but at a rather high instrument investment cost (Eskilsson and Björklund, 2000). Alternatively, microwave assisted extraction (MAE) offers a reduced extraction time and solvent consumption at a relatively moderate cost (Eskilsson and Björklund, 2000). Theoretically, the application of microwave on the sample induces ionic conduction and dipole rotation, leading to the intrinsic heating of the sample (Eskilsson and Björklund, 2000). When performed in closed-vessels, the extraction solvent achieves a temperature and pressure greater than those obtained using conventional techniques. The efficiency and speed of the extraction are subsequently enhanced. MAE has been successfully applied to the extraction of organic contaminants in a wide range of matrices such as vegetable and biological tissues, soils, sediments and water (Eskilsson and Björklund, 2000; Camel, 2000). In particular, MAE has been applied to the extraction of various POPs, including PCBs and organochlorine pesticides, in marine biota matrices (Carro et al., 2000; Vetter et al., 1998).

A literature search in the journal *Environmental Science and Technology* and amongst Elsevier publications using key words 'PCB' and 'tissue' restricted to year 2003 yielded 15 references in relation to POPs and marine biota, for which the extraction technique was described. Out of these 15 references, 8 reported the use of Soxhlet extraction. Other reported extraction techniques included centrifugation/homogenization (4), accelerated Soxhlet extraction (1), SFE (1) and sonication (1). This literature search reveals that most current environmental studies rely on old solvent and time consuming techniques, but only just one study reported the reduced use of organic solvents (i.e. SFE).

II – 2 – 3 Cleanup of biological tissue extracts

Removal of matrix interferences is a major challenge for POPs analysis in biological tissues. In particular, lipids are extracted together with the POPs and will affect the analysis if not removed (Strandberg et al., 1998). Current published cleanup steps for POPs in marine biological tissues include elution of the extract on a Florisil or silica gel column (Easton et al., 2002), a size-exclusion gel column (Jackson et al., 2001), a solid-phase extraction (SPE) cartridge (Jacobs et al., 2002), or treatment with concentrated sulfuric acid (Boon et al., 2002).

II – 2 – 4 Detection and quantification of POPs

Due to the large number of potential analytes present in the final extract (e.g. 209 PCB and PBDE congeners), further separation of the POPs of interest is required. GC has emerged as a tool of choice to separate analytes, based on their different boiling points and interactions with the stationary phase. The POPs of concern have a low polarity and are well separated on non- or slightly polar capillary GC columns (Frame, 1997a). Electron capture and selected-ion-monitoring mass spectrometry detectors are nowadays routinely used for POPs quantification due to their great sensitivity (Jones and de Voogt, 1999). Although electron capture detector achieves superior sensitivity (Frame, 1997a), mass spectrometry allows better confirmation on the identity of the analyte.

II – 2 – 5 Alternative techniques

Other techniques have been developed for the analysis of POPs in marine biological samples, although they have received less attention than the techniques presented above. For example,

ELISA immunoassay was successfully applied to PCB quantification in fish extracts as results were positively correlated with conventional quantification with gas chromatography (Zajicek et al., 2000). In another study, Schoeters et al. (2004) reported the use of CALUX bioassay, an *in vitro* luciferase reporter gene assay, for the measurement of dioxin-like activity in market fish samples.

II – 3 POPs in marine biota

II – 3 – 1 Transfer of POPs to the marine environment

The characterization of POP sources and their routes to the marine environment are complex and further investigation is required (Jones and de Voogt, 1999). Primary sources of organochlorine pesticides are generally their point of application. Leakage, leaching and volatilization during manufacturing, use or disposal of products appear as the primary source for industrial POPs. As an example, point sources of PCBs have been observed in the coastal waters adjacent to the Hawaiian Islands with a congener profile close to the commercial PCB mixture used in transformers (Miao et al., 2000). By-products, such as dioxins and furans, may be released by stationary industry plants or fuel combustion in vehicles (Vallack et al., 1998). Discharge of contaminated rivers into the ocean is also a source of POPs for the marine environment. Boon et al. (2002) showed, for example, that the River Tees, in UK East Coast, was a major source of tri to hexa-BDEs in the North Sea. Eventually, POPs spread at the regional and global scale through the processes of adsorption, volatilization and deposition (See Section II-1-5).

II – 3 – 2 Absorption, elimination and metabolism of POPs in marine biota

Pathways of exposure to POPs in marine organisms include direct uptake via gills or skin from the water, ingestion of particulates and consumption of contaminated food (van der Oost et al., 2003). The levels of POPs in marine organisms result from a complex combination of the exposure, uptake, elimination and metabolism processes, and therefore vary according to the lipophilicity of the contaminant, species, gender, breeding conditions, tissue composition and metabolic ability of the organism (Miao et al., 2000).

Marine organisms have the ability to metabolize POPs through processes such as hydroxylation or dechlorination (Herman et al., 2001; Kitamura et al., 1999). The liver is the organ most intimately involved in the biotransformation of lipophilic POPs into water soluble compounds that are more easily excreted (Bard, 1999; Marsh et al., 2004; van der Oost et al., 2003). However, metabolites can be more toxic than the parent contaminant (Bard, 1999; van der Oost et al., 2003). As a result of metabolization, it should be noted that concentrations of POPs in the organism do not necessarily reflect environmental levels. Therefore, levels of metabolites may also require consideration (e.g. DDT).

Biomagnification of the POPs levels is observed along the food chains, reaching peak concentrations in top predator species. Biomagnification is well documented for marine food webs of polar and temperate regions (Bard, 1999; Bayarri et al., 2001), but the literature for tropical zones is limited (Miao et al., 2000).

II – 3 – 3 Toxicological effects of POPs in marine biota

The toxicity of PCBs and organochlorine pesticides in aquatic organisms is well documented. The acute toxicity of POPs is generally greater for invertebrates and fish than mammals and ranges from moderate to very toxic (e.g. endrin) (UNEP, 2002b). A non-exhaustive list of chronic effects include hepatic carcinogenicity in fish (Chang et al., 1998), endocrine disruption in mussels (Binelli et al., 2001), immune system disorders in fish (Dunier and Siwicki, 1993) and reduction of fish egg viability (UNEP, 2002b).

In comparison, few data are available regarding the toxicological effects of PBDEs on the marine biota. Ethoxyresorufin-*O*-de-ethylase (EROD) assay is a common tool to assess *in vitro* toxicity of halogenated organic compounds, with reference to the highly toxic dioxins. In the review by De Wit (2002), several studies reports weak effects of EROD activity in fish larvae and mature fish liver. Other recent data also suggest that the dioxin-like activity of PBDEs is negligible in the environment (Chen and Bunce, 2003). Tomy et al. (2004) reported that dietary exposure of trout to PBDEs, at concentrations higher than environmental levels, was linked with changes in the thyroid activity. The potency of PBDEs as thyroid hormone disrupters has also been intimated for seals (Hall et al., 2004). This effect is presumably a consequence of the structural similarities between PBDEs, or their metabolites, and the thyroid hormone (T4) (McDonald, 2002).

II – 3 – 4 Bioindicators of marine contamination

Biological indicators, or bioindicators, are "organisms in which their characteristics reveal the presence or absence of environmental conditions that cannot be revealed by other species

or in the environment as a whole" (O'Brien et al., 1993). In the case of POPs and heavy metals, bioindicator species serve to evaluate the level of prevailing environmental contamination.

Ideally, a 'good' bioindicator of contamination should have the following features: (a) easy to capture, (b) in sufficient population density, (c) its distribution is well documented (d) the routes of exposure to the pollutant are known, (e) comparative data should be available in another area, (f) the relationship between biological parameters (e.g. gender) and pollutant load should be understood, (g) and the species should possess a good sensitivity to change of the pollutant load in its environment (Kaiser, 2001).

Bivalves, such as oysters and mussels, are filter-feeding organisms, so they readily accumulate POPs from their environment via the ingestion of waterborne organic particles. Consequently, bivalves have been widely used as a bioindicator species for various POPs including pesticides and PCBs (Phillips, 1985). The green mussel, *Perna viridis*, is naturally prevalent in Asia-Pacific waters and is considered as the best candidate as a bioindicator in South-east Asia (Phillips, 1985). Although *P. viridis* is cultured commercially in the Johore Strait which separates Malaysia from Singapore (Chou and Lee, 1997), there is no current monitoring of the marine environment in Singapore using green mussels. The Blue Mussel, *Mytilus edulis,* has been used for monitoring various POPs, and recently for PBDEs in Atlantic waters (e.g. Christensen et al., 2002). Mussels have shown a higher sensitivity to contamination than man-made indicators, such as semi-permeable membrane devices (Richardson et al., 2001).

Species/Location		DDT	chlordane	mirex	HCB	PCBs	PBDEs	Reference
Perna viridis								
Malaysia	ww	1.4±1.5	2.2±3.2	n.a.	0.01±0.01	1.3±0.5	n.a.	Monirith et al., 2000
Indonesia	ww	1.1±0.9	0.3±0.1	n.a.	0.01±0.01	1.5±0.3	n.a.	Monirith et al., 2000
Thailand	ww	5.6±8.1	1.2±1.4	n.a.	0.05±0.04	1.8±0.5	n.a.	Kan-atireklap et al., 1997
Phillippines	ww	1.7±1.2	3.7±3.7	n.a.	0.01±0.01	0.69-36	n.a.	Tanabe et al., 2000
Cambodia	ww	0.5±0.5	0.12±0.03	n.a.	0.02±0.01	<0.05-5.1	n.a.	Monirith et al., 2000
Vietnam	ww	44±111	0.3±0.4	n.a.	0.01±0.02	1.1±0.4	n.a.	Monirith et al., 2000
India	ww	11±10	0.5±0.5	n.a.	0.06±0.11	0.31-15	n.a.	Tanabe et al., 2000
Hong Kong	dw	26-47	0.6-3.8	n.a.	n.a.	119-415	n.a.	Richardson et al., 2001
China	dw	14-640	0.04-6.4	n.a.	0.01-13	1.3-13	n.a.	Fung et al., 2004
Various species								
Netherlands	ww	n.a.	n.a.	n.a.	n.a.	n.a.	1.3	De Boer and Cofino, 2002
Denmark	ww	n.a.	n.a.	n.a.	n.a.	n.a.	0.08-0.81	Christensen et al., 2002
Baltic Sea	ww	n.a.	0.06-0.09	BLD	n.a.	n.a.	n.a.	Falandysz et al., 2001
Mediterranean Sea	dw	16-550	n.a.	n.a.	BLD-1.9	50-3500	n.a.	Villeneuve et al., 1999
Americas	dw	9.1-960	2.8-9.6	n.a.	n.a.	10-3800	n.a.	Sericano et al., 1995

Table II-3: Concentrations of POPs reported in the literature for *P.viridis* and other mussel species (ng/g). ww: wet weight. dw: dry weight. n.a.: non available.

II – 4 Implication for humans

II – 4 – 1 POP exposure for humans

The routes of exposure to POPs for human beings include inhalation, dermal exposure and ingestion of water or food contaminated by POPs. Cases of occupational or accidental exposure to POPs have been recorded, particularly during the application of pesticides (Vallack et al., 1998). However, food consumption is one of the most important pathways of exposure for the general population (Dougherty et al., 2000; Stefanelli et al., 2004).

Among food items, seafood has been identified as a major source of POPs (Bocio et al., 2003), particularly in Asian countries, where fish and shellfish account for a significant component of the dietary intake (Kannan et al., 1997; Ohta et al., 2002; Simmonds et al., 2002). As a consequence of high consumption rates, a strong correlation exists between the levels of PBDEs in human breast milk and fish consumption (Ohta et al., 2002; Meironyté et al., 1999). Most controversially, in the USA, it has been shown that 11-years old children born to women who had consumed contaminated fish from the Great Lakes, i.e. exposed to polychlorinated biphenyls, had memory and attention problems (Jacobson and Jacobson, 1996).

Recently, assessment of POPs in farmed fish has raised concerns, where higher levels of POPs have been recorded in edible tissues relative to wild fish (Antunes and Gil, 2004; Easton et al., 2002; Hites et al., 2004). As a result, the risk assessment by Hites et al. (2004)

advised to limit the consumption of farmed salmon no more than once a month, whereas wild species could be consumed between 1 to 8 times per month.

II – 4 – 2 Toxicological effects of POPs in humans

The acute toxicity of POPs to humans ranges from moderate (e.g. DDT) to lethal (e.g. aldrin) (UNEP, 2002b). Potential health effects of POPs include developmental and neural effects, immunotoxicity and teratogenicity (UNEP, 2003; Vallack et al., 1998). Dioxins, furans, HCB, some PCBs, chlordane, mirex, toxaphene and DDT are considered carcinogenic or potentially carcinogenic (UNEP, 2003). Kalantzi et al. (2003) also showed recently that environmental levels of HCH can induce alterations on human prostate and breast cells. The toxicity of PBDEs to humans has not been clearly demonstrated to date. However, available data suggest that PBDEs are potentially carcinogenic and may cause thyroid hormone disruption and neurobehavioral deficits (McDonald, 2002).

II – 2 – 3 Safety standards

Maximum residue limits (MRLs) for heavy metals and POPs in Singapore (Government of Singapore, 1990) and the United States (USFDA, 2001) are presented in Table II-4. Monitoring programs conducted in the US have revealed that POPs levels in food are frequently above threshold limits (Dougherty et al., 2000; Greenfield et al., 2000)

Contaminant	Singapore	US FDA
chlordane	0.05 μg/g	0.3 μg/g
heptachlor		0.3 μg/g
heptachlor epoxide		0.3 μg/g
mirex		0.1 μg/g
DDTs	5.0 μg/g	5.0 μg/g
PCBs		2 μg/g

Table II-4: Maximum residue limits for POPs in seafood in Singapore and the United States

II – 5 Towards a better understanding of POPs in South-east Asia

II – 5-1 POPs in South-east Asia

In 1993, Asia was declared the major source of POPs in the global environment (Iwata et al., 1993). Despite the importance of the tropical region as a source of POPs in the global cycling of these compounds, large data deficiencies exist on the sources, ecotoxicology, toxicology and transport of such pollutants in the Asian environment (UNEP, 2002a; 2002b).

All POPs of concern are officially banned in most South-east Asian countries (UNEP, 2002b). However, regulations regarding POP usage are recent in some cases; e.g. chlordane was banned in 1998 in Malaysia, and 1999 in Singapore (UNEP, 2002b). DDT, mirex, endosulfan and HCHs have been used in South-east Asia in the last 10 years (UNEP, 2002b). The use of pesticides, such as DDT, in tropical countries is often justified for the eradication of disease vectors such as the malaria-carrying mosquito and countries, such as Philippines, allow spreading of DDT in restricted use.

Studies on the environmental behavior of PBDEs are chiefly derived from Europe, North America and the Arctic; for Asia, only data from Japan has been reported (De Wit, 2002), and recently by Ueno et al. (2004) for tuna in the China Sea. Asia is an important consumer of octa-BDEs and deca-BDEs, although its use of penta-BDEs is reported to be negligible (De Wit, 2002). No data are available on the occurrence of PBDEs in South-east Asia prior to the completion of the present study.

III – 5-2 POPs in Singapore

Singapore is located in Southeast Asia, approximately 120 km north of the equator, at the southern tip of the Malaysian peninsula with Indonesia to the south (Figure II-2). From a maritime perspective, the island state of Singapore is at the interface of the Straits of Malacca and the South China Sea.

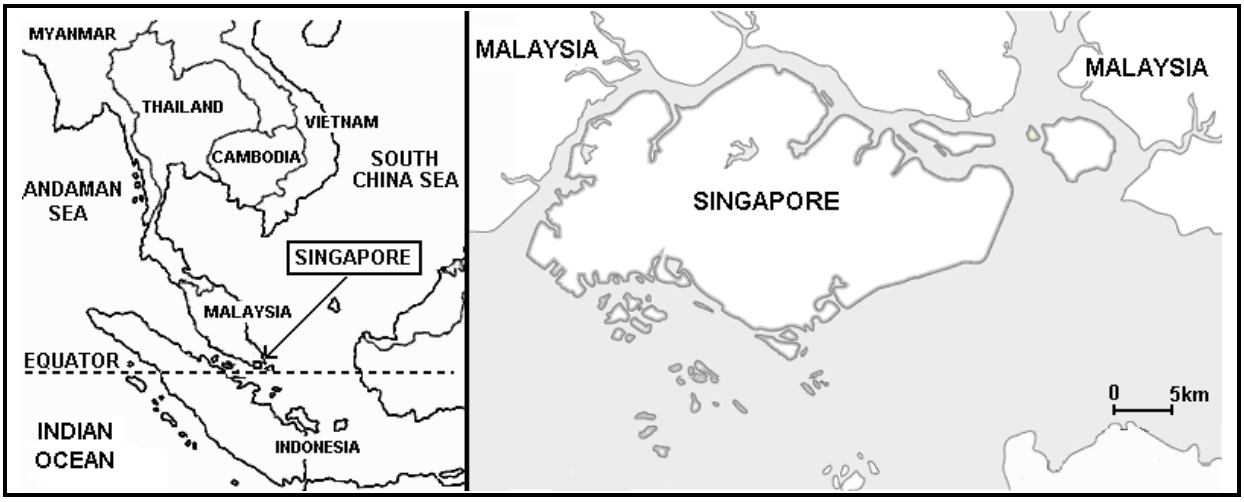

Figure II-2: Geographical location of Singapore (a) and map of Singapore (b).

This strategic geographical location has resulted in Singapore becoming one of the most vibrant ports in the world, with an annual freight shipping tonnage in excess of 313 million

tonnes (Economic Department, 1999). Singapore's environment is highly urbanized and densely populated, reaching 4.13 millions in 2001 (http://www.singstat.gov.sg/). Although highly industrialized, Singapore still has a wide diversity of marine habitats including sandy beaches, mangroves, rocky shores and coral reefs. However, many of these habitats are threatened by land reclamation and intense port activity, as well as marine pollution (Thia-Eng et al., 2000).

All POPs are now banned in Singapore, as presented in Table II-5 (UNEP, 2002b). However, literature available data on POPs in Singapore is scarce before 2000. In the 1990's, DDT residues have been detected in human blood serum in Singapore and partly attributed to seafood consumption (Luo et al., 1997). Some data for POPs in seawater are available after 2000 in Basheer et al. (2002a; 2003). Simultaneously to the present study, other scientists at TMSI conducted work on the analysis of POPs in marine sediments and sea surface micro-layer (Wurl and Obbard, 2004a; 2004b). No baseline study for POPs in the Singapore's marine biota is reported in the scientific literature.

Year of ban	Compound
1980	PCBs
1985	aldrin, dieldrin, endrin, DDT, heptachlor, HCB, toxaphene, mirex
1999	chlordane
Not banned	PBDEs

Table II-5: Date of ban on POP usage in Singapore (UNEP, 2002b).

CHAPTER III – GENERAL MATERIAL AND METHODS

This chapter describes the general experimental materials and methods used in the study. Specific details are provided in subsequent individual chapters.

III – 1 Chemicals

All chemicals used for POPs analysis were of high purity, unless otherwise stated. In particular, solvents, silica gel and anhydrous sodium sulfate were of pesticide grade, i.e. confirmed to be pesticide-free by the various suppliers (Aldrich, St. Louis, MO, USA; Fisher, Hampton, NH, USA; Tedia, Fairfield, OH, USA; Reagent Chemical Industry, Bangkok, Thailand). Silica gel (70-230 mesh) was activated at 450 ºC for 12 h prior to use. High purity nitrogen and helium gas (>99.9995%) were purchased from Soxhal (Singapore). ^{13}C-labelled PCB standards were purchased from Promochem (Welwyn Garden City, U.K.). PCB and organochlorine pesticides standards were obtained from QMx (Thaxtet, U.K.), Accustandard (New Haven, CT, USA) or Dr Ehrenstorfer (Augsburg, Germany). PBDE standards were obtained from QMx (Thaxtet, U.K.) or Accustandard (New Haven, CT, USA).

III – 2 Standard reference materials

Standard Reference Materials (SRMs) - mussel tissue SRM 2978- and -cod liver oil SRM 1588a- were obtained from National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

III – 3 Preparation of biological tissue samples

III – 3 – 1 Sample preparation

All stainless steel and glass equipment used for the preparation and storage of glassware was cleaned with detergent solution and rinsed with solvents prior to use. Organisms collected in the field were transported to the laboratory in polyethylene bags in ice-boxes and processed within 24 hours of collection. Biological tissues of interest were extracted and homogenized in a Waring 7011S stainless steel blender (Torrington, CT, USA). Homogenated samples were stored in the dark at -20°C. A maximum holding time of one year is advised by the USEPA (1999) for PCB determination, although it is stated that no loss in quality is noted after this period.

III – 3 – 2 Determination of moisture content

Tissue moisture content was determined using one of the two following methods:

- Gravimetric comparison before and after freeze-drying of sample using a Virtis Advantage freeze-dryer (Gardiner, NY, USA).
- Measurement with a Mettler Toledo LJ16 Moisture Analyzer (Greifensee, Switzerland). Moisture content is derived from the weights before and after heating the sample with infrared light.

III – 4 POPs extraction in marine biological tissues

III – 4 – 1 Sample preparation

Freeze-dried samples were directly weighed in the extraction vessel (+/- 1 mg precision). In the case of wet tissues, an accurate weight of pooled tissue sample was mixed thoroughly with anhydrous sodium sulphate (ratio 1:5) prior to POPs extraction.

III – 4 – 2 Soxhlet extraction

The Soxhlet apparatus was rinsed prior to extraction for 2 h with 200 mL of acetone/DCM (1:1, v:v). Soxhlet extraction was performed for 8 h with either 200 mL of acetone/DCM (1:1, v:v) or n-pentane/DCM (1:1, v:v) (Wu et al., 2001; Carro et al., 2000).

III – 4 – 3 Accelerated Soxhlet extraction

Accelerated Soxhlet extraction (ASE) uses the same principle as conventional Soxhlet with the additional feature whereby the extraction thimble is also heated, thereby accelerating the whole extraction process. ASE was performed with a BUCHI B-811 (Buchi Labotechnik, Flawil, Switzerland) rinsed for 2 hours with 200mL of acetone/DCM (1:1, v:v) prior to extraction. One gram of sample was extracted for 4 h with 200 mL of n-hexane/DCM (3:1, v:v) (Moisey et al., 2001).

III – 4 – 4 Microwave assisted extraction

Microwave assisted extraction (MAE) was performed in a Mars X microwave oven (CEM, Matthews, NS, USA). Samples were extracted in a PTFE vessel with a mixture of n-pentane/DCM (v/v 1:1). Details on the amounts of tissue, the volume of solvent are given in the subsequent following chapters depending on the application. The oven was programmed for a temperature increase to 115°C over 10 min which was then maintained for 15 min. The optimization and choice of MAE parameters are discussed in Chapter IV.

III – 4 – 5 Gravimetric determination of lipids

Lipid content was determined gravimetrically on part or the whole solvent extract, depending on the level of precision required. Small solvent volumes were dried under a gentle flow of purified nitrogen. Larger volumes were dried using a Laborota 4000 rotary evaporator (Heidolph Instruments, Schwabach, Germany). This method has been validated elsewhere for Soxhlet extraction (Wu et al., 2001; Binelli et al., 2001).

III – 5 Sample cleanup for POPs analysis

III – 5 – 1 Acid silica gel chromatography

Lipids in the extract were first degraded and partially removed on a 15 g acid silica gel column (silica gel/ H_2SO_4 98% (w/w 2:1)) eluted with 150 mL of n-hexane. For samples with low lipid content, 10 g of acid silica gel and 100 mL of n-hexane were used. This type of chromatography makes use of the adsorption properties of molecules to separate the analytes from some matrix interferences. Concentrated sulphuric acid degrades most of the matrix

interferences. Most of the POPs are not affected by sulphuric acid; however methoxychlor and dieldrin are known to be degraded by the acid, and endrin is transformed into endrin ketone (Di Muccio et al., 1990; Manirakiza et al., 2002). Effectiveness of the lipid degradation was acceptable when the colour in the column (i.e. degradation products) did not elute lower than two thirds of the column length. Samples were then concentrated to 1 mL using rotary evaporator before gel permeation chromatography.

III – 5 – 2 Gel permeation chromatography

Remaining lipids and other impurities in the elute were then completely removed by gel permeation chromatography (GPC) using 6 g of Biobeads SX-3 (Bio-Rad Laboratories, Hercules, CA, USA) per column eluted with n-hexane/DCM (v/v 1:1). The first 16 mL of the GPC elute was discarded and the following 30 mL was collected and concentrated to 0.5 mL using rotary evaporation. GPC is a size exclusion chromatographic technique, i.e. molecules with different sizes will elute at different speed in the column. Smaller molecules, for example, elute later than larger ones as their mean path length in the column is longer. Extracts were further concentrated to 50 μL under a gentle flow of purified nitrogen using 50 μL of dodecane containing the internal standards (Refer to Section III-7-5, Chapter III). This method was adapted from Thomas et al. (1998).

III – 6 Gas chromatography-mass spectrometry

Except for green mussel samples analyzed in Lancaster University (See Chapter V), the analysis and quantification of pesticides, PCB and PBDE congeners was performed using a Shimadzu QP-2010 (Shimadzu Asia-Pacific, Singapore) gas chromatograph coupled with a

mass spectrometer (GC-MS). Compounds were separated on a DB-5ms (J&W Scientific, USA) capillary column (30 m length, 0.25 mm internal diameter) with a gas flow of helium at 35 $cm.min^{-1}$. Three different GC oven programs were used during the course of the experiments:

- Program 1: 50°C held for 2 min, 20°C/min to 140°C, 4°C/min to 290°C held for 5 min.
- Program 2: 50°C held for 1 min, 20°C/min to 150°C held for 5 min, 3°C/min to 250°C, 10°C/min to 300°C held for 10 min.
- Program 3: 170°C held for 11 min, 3°C/min to 250°C held, 10°C/min to 300°C held for 10 min.

The detector was operated in electron impact (EI) mode with Selected Ion Monitoring (SIM). Masses and retention time for each contaminant are presented in Appendix A. Analyte quantification was performed using a seven-point linear calibration (r>0.99) with diluted standards. Except when specified otherwise, DDTs refer to the sum of the parent *p,p'*-DDT and its metabolites *p,p'*-DDD and *p,p'*-DDE, chlordanes to the sum of γ- and α-chlordane, and PCBs to the sum of congeners 17, 18, 28/31, 33, 44, 49, 52, 70, 74, 82, 87, 90/101, 95, 99, 105, 110, 118, 128, 132, 138, 149, 151, 153, 156, 169, 170, 171, 177, 180, 183, 187, 194, 199, 201, 205, 206, 208 and 209. These congeners were selected due to their toxicity, abundance and persistence in the environment (Jones, 1998).

III – 7 Quality assurance for POPs analysis

III – 7 – 1 Spiking of recovery standards

Recovery standards were spiked into samples before extraction to determine any loss of analytes during the whole analytical procedure. ^{13}C-labelled PCBs are routinely used as recovery standards for POPs analysis (e.g. Kumar et al., 2001; Moisey et al., 2001, Strandberg et al., 1998). Non-labelled PCBs are also used as recovery standards in POPs analysis (e.g. Datta et al., 1999; Moisey et al., 2001; Stapleton et al., 2001) provided they do not occur in the environmental matrices. PCB 55 and 61 have not been reported to date in any environmental samples (Martin et al., 2003), and only traces (<0.1% in weight) are reported in the PCB commercial mixtures of Aroclor (Frame, 1997b). ^{13}C-labelled PCBs (i.e. congeners 28, 52, 101, 138, 153 and 180) or non-labelled PCBs (i.e. congeners 55 and 61) were therefore used in this study as recovery standards.

III – 7 – 2 Analysis of standard reference materials (SRMs)

SRMs are extensively used to check the precision and accuracy of analytical methods. Mussel tissues -SRM 2978- or cod liver oil -SRM 1588a- were analyzed to validate analytical methods for PCBs and organochlorine pesticides. However, it is worth noting that there is currently no available SRM for PBDEs (Alaee et al., 2001).

III – 7 – 3 Procedural blanks

A procedural blank, consisting in 20 g of anhydrous sodium sulphate, was extracted, cleaned and analyzed the same way as other samples with every set of ten samples (Stapleton et al.,

2001). The method detection limit (MDL) was calculated as the average of the procedural blank peak area plus three times the standard deviation (El Jarrat et al., 2002; Voorspoels et al., 2003). Average blank values were subtracted from sample values (Dodder et al., 2002).

III – 7 – 4 Replication

Samples analysis was performed in duplicate, unless otherwise specified. In some cases, batches of samples were analyzed 3 to 5 times in order to assess the homogeneity of samples.

III – 7 – 5 Quality assurance for GC-MS analysis

Internal standards were used for the POPs quantification and prepared in the dodecane keeper used for the final extract. Internal standards included pentachloronitrobenzene (PCNB), ^{13}C-labelled PCB 141, non-labelled PCB 30 and 62 (Hale et al., 2001; Moisey et al., 2001). Non-labelled PCBs are also used as internal standards in POPs analysis (e.g. Moisey et al., 2001) provided they do not occur in the environmental matrices. PCB 30 and 62 have not been reported to date in any environmental samples (Martin et al., 2003), and only traces (<0.02% in weight) are reported in the PCB commercial mixtures of Aroclor (Frame, 1997b).

During the integration of the GC-MS spectrum, analytes with a difference in the retention time of more than 0.1 min relative to the standards were not quantified. For each analyte, a minimum of two ions were monitored by GC-MS (See Appendix A). Peaks with a primary to secondary ion ratio of more than a 15% variation from that of the standards were not quantified (derived from Dodder et al., 2002).

III – 8 Statistical analysis

All statistical data analyses were performed using XSTAT 6.19 software (Addinsoft, Brooklyn, NY, USA).

III – 8 – 1 Comparison of population medians

The Mann-Whitney U test was performed to test the hypothesis of the supposed superiority or equality of two population medians. To compare differences between more than two populations, data were assessed using the Kruskal-Wallis test. Significance level was set to *p*-value of 0.05.

III – 8 – 2 Principal component analysis (PCA)

PCA creates new orthogonal variables, called principal components, resulting from the linear combination of the columns of the data matrix. Using the first and second principal components, it is possible to detect similarities amongst the set of data. The effectiveness of PCA of multivariate data sets for environmental issues has been described by Zitco (1994).

PCBs and PBDEs are sold commercially as technical mixtures, each with a specific congener pattern. Congener profiles have been determined for Aroclor mixtures 1221, 1232, 1242, 1248, 1254, 1260 and 1262 (Frame, 1997b), and for commercial pentabrominated diphenyl ethers mixtures Bromkal 70-5DE and DE-71 (Sjödin et al., 1998; Dodder et al., 2002). PCA was used in this study to compare the relative PCB and PBDE congener profiles of biota samples to those of known commercial mixtures. This provides insight into the potential source of PCB exposure in environmental samples.

III – 9 Good laboratory practices

III – 9 – 1 Chemicals handling

As discussed in the Section II-4-2, Chapter II, POPs are toxic to human health, and can be carcinogenic in particular. POPs standards were kept in sealed containers in a fridge dedicated for chemical storage. Personal protection equipment included use of lab coat, safety goggles and respiration mask. Nitrile gloves were worn when organic solvents were used whereas latex gloves were used when using acids.

Table III-1 presents the threshold and short-term exposure limits for the various solvent used. The solvents used in this study are less toxic than benzene or acetonitrile, but necessitate good ventilation. Solvent and solid wastes (e.g. used acid silica gel and extracted samples) were collected for sending to a chemical recycling company.

Organic Solvent	TLV (ppm)	STEL
acetone	1000	1000 ppm / 30 min
dodecane	n.a.	n.a.
dichloromethane	500	100 ppm / 60 min
n-hexane	500	500 ppm / 30 min
n-pentane	500	n.a.

Table III-1: Threshold limit values (TLV) and short term exposure limit (STEL) for different organic solvents (Cheremisinoff, 1999). n.a.: not available.

III – 10 – 2 Equipment safety

The use of microwave assisted chemistry can result in the production of high pressure inside extraction vessels. To avoid the risk of explosion, the vessels are equipped with rupture membranes which explode at 200 psi.

GC-MS equipment does not represent any major hazard. To avoid emission of contaminants in ambient air, an active carbon filter was installed on the rotary pump.

III – 10 – 3 Handling of living organisms

All living organisms were treated humanely accordingly to institutional guidelines, with due consideration for the alleviation of discomfort. As an example, organisms were put to sleep in ice or in freezer at -20°C to provoke a quick non-painful death.

CHATER IV – MICROWAVE ASSISTED CHEMISTRY APPLIED FOR THE DETERMINATION OF POPs IN MARINE BIOLOGICAL TISSUES

IV – 1 Introduction

Current published methods for the detection of PBDEs in marine tissue matrices include Soxhlet extraction (Christensen et al., 2002; Akutsu et al., 2001), column elution (Dodder et al., 2002; Alaee et al., 2001), a manual shaking procedure (Ikonomou et al., 2002) and a pressurized liquid extraction technique (Hale et la., 2001). Vetter (2001) reported the use of MAE to extract PBDEs from several marine biological matrices, but no performance or validation of the technique was conducted.

PBDEs and PCBs are known to have a similar behavior during sample extraction, cleanup and gas chromatography quantification (ElJarrat et al., 2003). The aim of the present study was to determine optimum MAE parameters for conventional POPs analysis in marine biota matrices and determine whether the optimized MAE method can also be applied to PBDE analysis. Method performance was evaluated for the PBDE congeners 47, 99 and 100 due to their environmental occurence (De Wit, 2002; Akutsu et al., 2001). Validation of the whole analytical procedure involved the analysis of procedural blanks, the recovery of PBDEs spiked in various marine biological matrices, and a comparison with the well-established Soxhlet extraction procedure using standard reference materials.

Lipid content determination is an important step during POPs analysis, including PBDEs, as levels in marine biota tissues are often reported on lipid weight basis (e.g. Bayarri et al., 2001; Dodder et al., 2002). Lipid content is conventionally measured gravimetrically after Soxhlet extraction. However, there is only one report on the use of MAE for the determination of lipid content, but no comparison was presented to the Soxhlet method (Mooibroek et al., 2002). The present study also investigates the possibility of using MAE to determine sample lipid content.

IV – 2 Materials and Methods

V – 2 – 1 Choice of MAE parameters

Various solvent mixtures are reported in the literature for the analysis of PCBs and OCPs in specific marine biological matrices, including n-pentane-DCM (1:1, v/v) (Carro et al., 2000) and acetone-hexane (4:1; v/v) (Jayaraman et al., 2001). These mixtures were preliminary assessed using 1 g of SRM 2978 and comparing recoveries with certified values.

Optimun extraction solvent volume is essential for proficient extraction (Camel, 2000). For highest efficiency, the entire sample must be submerged, with volumes used ranging from 10 to 30 mL in the literature (Vetter et al., 1998; Eskilsson and Bjorklund, 2000; Jayaraman et al., 2001). In the present study, 25 mL of solvent was required to completely submerge the samples. From previous studies, optimal analyte extraction was achieved in the range of 90 to 120°C (Eskilsson and Bjorklund, 2000; Camel, 2000; Jayaraman et al., 2001), and a temperature of 115°C was therefore selected in the present work (adapted from Carro et al.,

2000). Finally, the extraction time was set to 15 min (Jayaraman et al., 2001; Carro et al., 2000).

IV – 2 – 2 GPC elution profile

There is no report in the literature of the typical analyte profile from a GPC column for PBDEs. In this study, the profile was determined by elution of a standard containing both OCPs and PBDEs with n-hexane-DCM (1:1, v/v) on 6 g of Biobeads SX-3, and then analyzing each 1.9 mL fraction from the column separately. Similarly, the typical elution profile for lipid from the GPC column was determined by elution of 50 mg of candle wax diluted in 1 mL of hexane. Using this procedure the appropriate solvent volumes to be discarded and collected during the GPC cleanup procedure could be determined.

IV – 2 – 3 Spiked blank samples

Ten procedural blank samples (20 g of anhydrous Na_2SO_4), spiked with the recovery standards, were extracted and analyzed using the optimized method to calculate the method detection limit (MDL).

IV – 2 – 4 Spiked sample PBDE recovery test

Muscle tissues of salmon and conger eel, liver tissues of sea bass and the whole soft tissues of the green mussel *Perna viridis* were used for the PBDE recovery test. The four matrices chosen in our study cover a wide range of moisture and lipid contents, as detailed in Table IV-1. Tissues were homogenized in a stainless steel blender. The sample size was chosen based on the lipid content of the tissues and the expected concentration in the matrix. An

adequate sample size was fixed to 4 g for fish muscles and green mussel tissues. As liver tissues usually contain more lipids and higher levels of organic pollutants (De Wit, 2002), only 2 g were used for analysis.

Marine organism	Matrix	Moisture content (%)	Lipid content (%)
Conger eel	Muscle tissues	78	1.2
Salmon	Muscle tissues	67	8.2
Green mussel	Whole soft tissues	68	3.3
Seabass	Liver tissues	48	38.5

Table IV-1: Characteristics of the four types of marine biological tissues used for the recovery test.

The recovery test was set to target concentrations ranging 50 to 200 ng/mL in the final extract for each PBDE congener analysed. This range of concentration permitted the quantification of congener levels usually recorded in biota samples (De Wit, 2002). Samples were spiked with 5, 7.5, 10, 15 and 20 µL of a standard solution measured at 535 ng/mL, 592 ng/mL and 604 ng/mL respectively for BDE-47, 99 and 100. The spiking solution was allowed to equilibrate with the sample for 2 hours before extraction.

IV – 2 – 5 Standard reference materials

To date, there is no SRM data available for PBDE analysis in marine biological tissues. The analytical method validated in this study was used to quantify PBDE concentrations in mussel tissue SRM2978 and cod liver oil SRM 1588a. These SRMs have certified

concentrations for OCPs and PCBs, and these compounds were also quantified to compare PBDE recovery using MAE compared to conventional Soxhlet extraction.

IV – 2 – 6 Chemical analysis

Blanks, tissue samples and SRMs were spiked with PCB 55 and 61 surrogate standards to report on analyte loss. These samples were analyzed using the optimized MAE with 25 mL of n-pentane-DCM (1:1, v/v) as the extraction solvent. After extraction, cleanup and analysis were performed as described in Sections III-5 and III-6, Chapter III.

IV – 2 – 7 Gravimetric determination of tissue lipid content

Lipid content was determined gravimetrically following both Soxhlet and MAE extraction over twelve biological matrices. The samples were chosen amongst both seafood and meat products to create a wide range of lipid content. Samples included: green mussel soft tissues; shrimp and crab meat; white snapper, cod and salmon fillet, fish paste, minced pork, beef and chicken, and chicken and pig liver. Samples were purchased from a local supermarket. Two batches were created per tissue type using samples collected at different times. Six and 4 g of tissues were used for Soxhlet extraction and MAE respectively. Extracts were dried using a rotary evaporator and the residue was weighed accurately (+/- 1 mg precision). Samples were extracted with 25 mL and 150 mL of n-pentane/DCM (v/v 1:1) for MAE and Soxhlet respectively.

IV – 3 Results

IV – 3 – 1 Choice of the solvent

Analyte recoveries compared to certified values for PCBs and OCPs in SRM 2978 with MAE reached, respectively, 80±22% for *n*-pentane-DCM (1:1, v/v) and 69±42% for acetone-hexane (4:1; v/v) (See Table IV-2). MAE using *n*-pentane-DCM gave results comparable to Soxhlet, with a slightly lower average recovery for PCBs. As a result, *n*-pentane-DCM (1:1, v/v) was chosen as the MAE mixture for the rest of the project.

Compound	**Recovery MAE (%)**		**Recovery Soxhlet**
	acetone-hexane	**n-pentane-DCM**	**(%)**
α-chlordane	78	95	89
p,p'-DDE	49	96	75
p,p'-DDD	59	87	79
PCBs[a]	71	77	91
Average recovery ± SD	69%±42%	80%±22%	89%±26%

Table IV-2: Comparative recoveries of OCPs and PCBs for MAE and Soxhlet extraction of SRM2978. [a] average recovery for PCB congeners 18, 28, 31, 44, 49, 52, 66, 90, 95, 99, 110, 118, 138, 149, 151, 153, 156, 170, 180, 183.

IV – 3 – 2 Extraction temperature and pressure

The typical temperature and pressure profile inside the extraction vessel during MAE of 4 g of tissues with 25 mL of n-pentane-DCM (1:1, v/v) is presented in Figure IV-1. Once the plateau is reached, the temperature and pressure averaged 115±1°C and 137±6 psi (i.e. 10 bar). Therefore, the solvent/sample mixture can be assumed to attain a constant temperature and pressure during extraction.

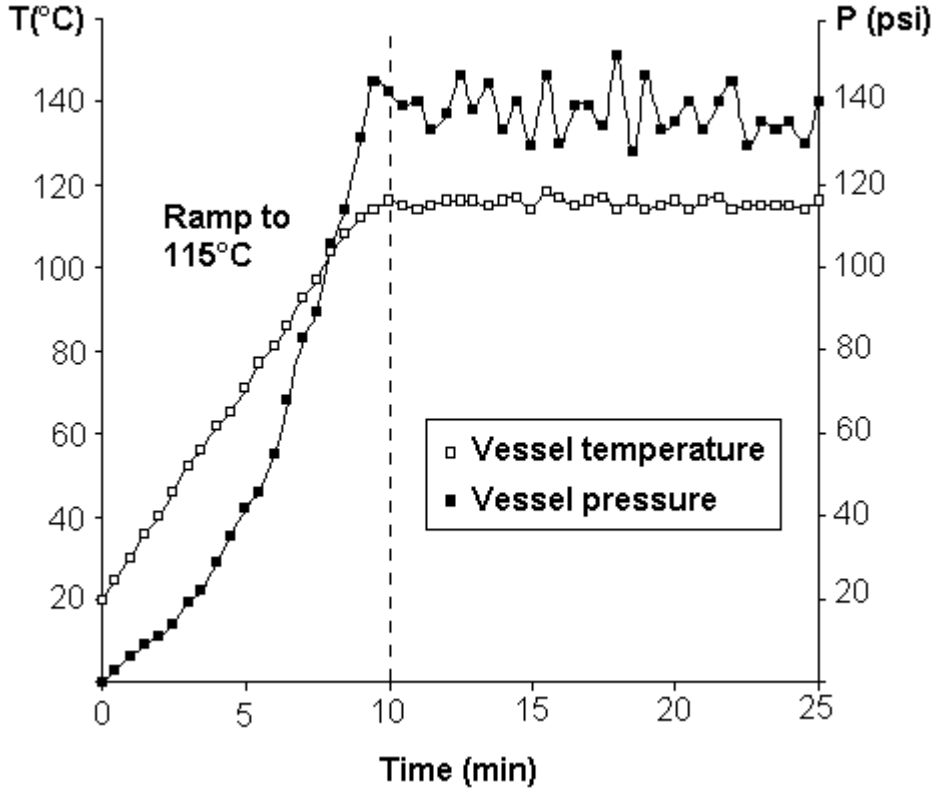

Figure IV-1: Typical temperature and pressure profile inside the extraction vessel during MAE of 4 g of tissue with 25 mL of n-pentane-DCM (1:1, v/v).

IV – 3 – 3 Extract cleanup

A typical analyte profile from the GPC column is shown in Figure IV-2. PBDE congeners 47, 99 and 100 elute between 18 and 25 mL. The profile is similar to OCPs such as DDTs and chlordanes. HCHs elutes later, between 20 and 30 mL. Large lipid molecules elute between 5 and 14 mL. Therefore, the first 16 mL fraction, containing lipids, was discarded.

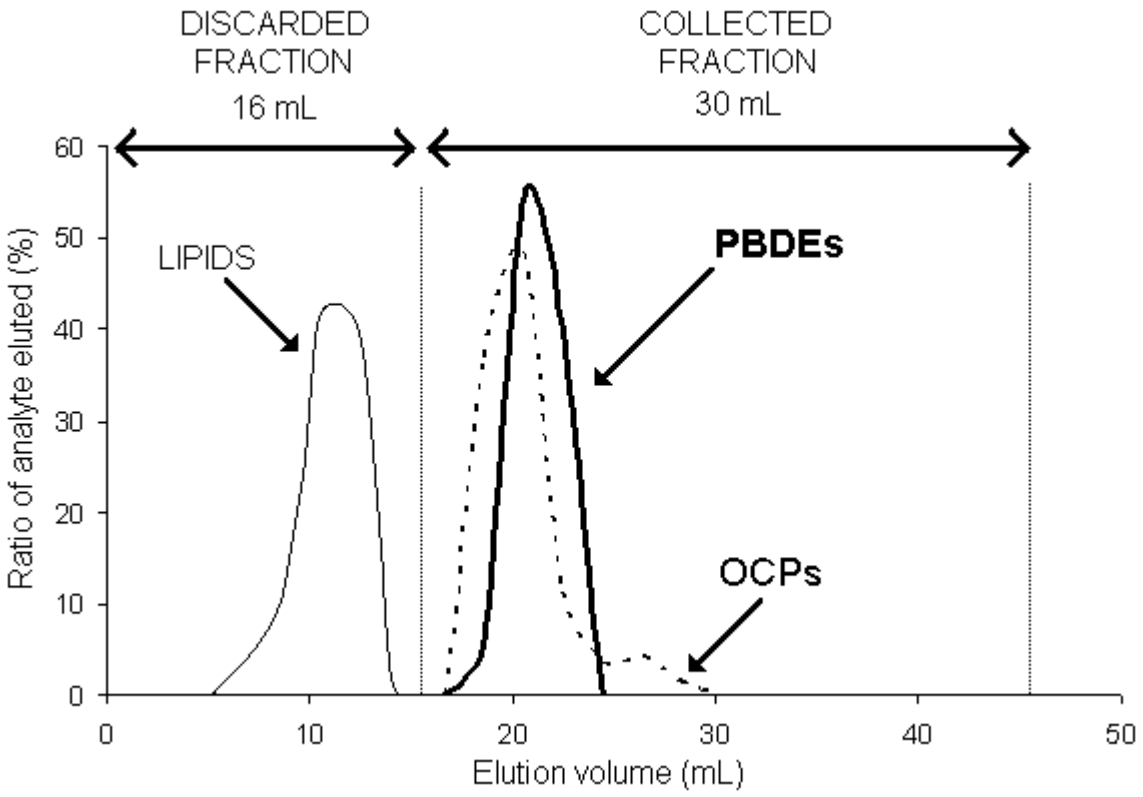

Figure IV-2: Elution profile for PBDEs (i.e. BDE-47, 99 and 100) and major OCPs (i.e. aldrin, dieldrin, endrin, HCHs, *p,p*'-DDT, *p,p*'-DDE, *p,p*'-DDD, α- and γ-chlordane, endosulfan, metoxychlor, heptachlor and heptachlor epoxide) with 6 g of Biobeads SX-3 column using n-hexane/DCM (v/v 1:1) as a mobile phase.

A chromatogram showing the quantification ion of the PBDE congeners in a 200 ng/mL mixed standard is shown in Figure IV-3a. Chromatograms obtained for SRM1588a and SRM 2978 extracted with MAE are presented in Figures IV-3b and 3c, respectively. The spectra do not show any major interference for either the quantification or confirmation ions. Therefore, the extract clean-up procedure can be considered as effective.

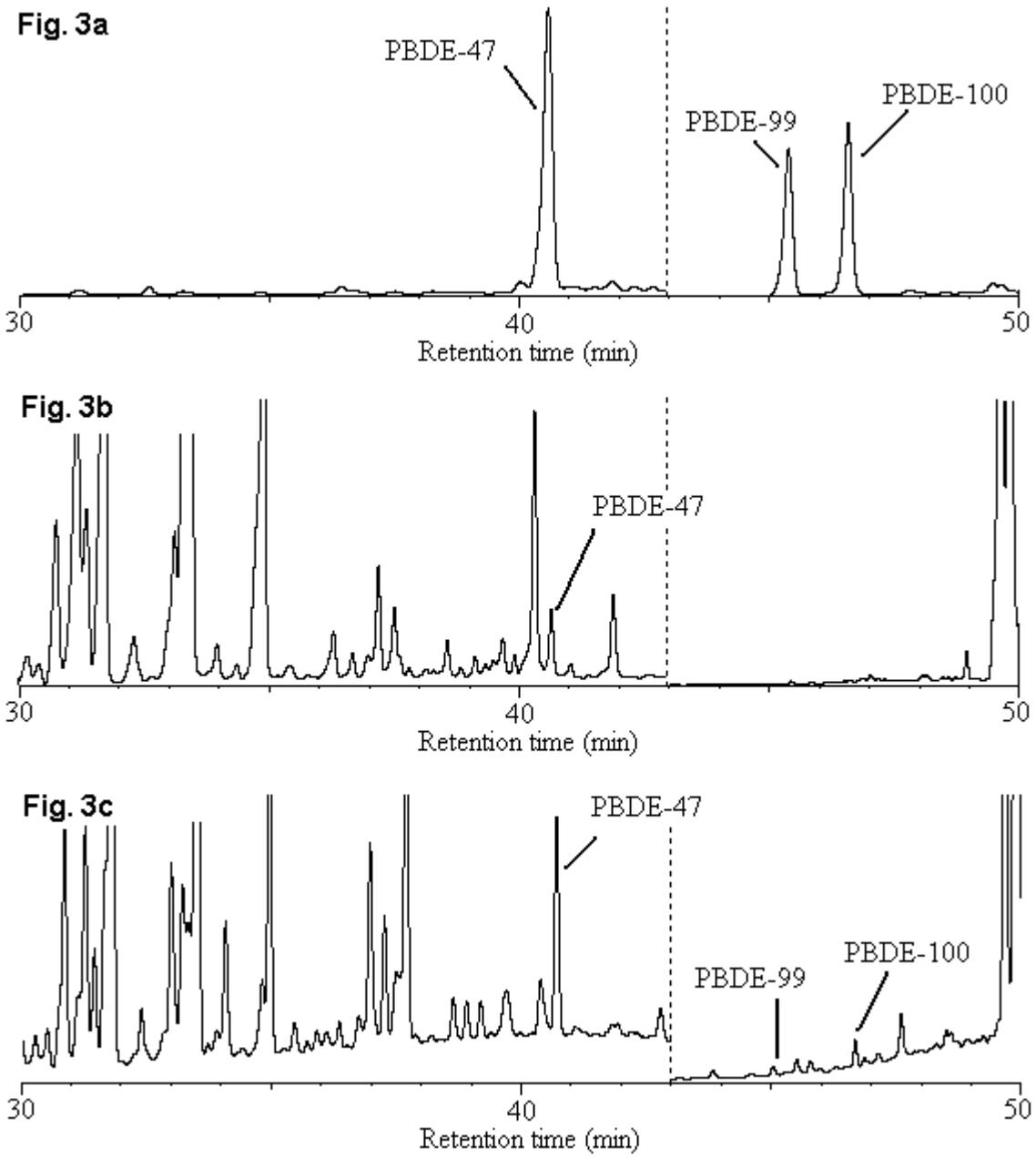

Figure IV-3: Comparative chromatograms for the quantification ion of the PBDE congeners (47, 99 and 100) for a 200 ng/mL standard (Fig 3a) and for SRM1588a (Fig. 3b) and SRM 2978 (Fig. 3c), extracted using MAE.

IV – 3 – 4 Surrogate recovery

Quantitative results are deemed acceptable provided surrogate compounds are recovered in the range of 70-130%, according to USEPA Method 1688a (1999) for PCB congeners in

marine biota tissues. Actual recoveries for PCB 55 and PCB 61 averaged 95%±12% and 93%±12% respectively, confirming that there was no unacceptable loss of analytes during the entire analytical procedure.

IV – 3 – 5 Calibration curves, MDLs and secondary ion for PBDEs

Linear calibration curves were obtained for PBDE congeners 47, 99 and 100 over the 0 to 200 ng/mL range, with an r^2 coefficient of greater than 0.99. The MDL was below 0.05 ng/g of sample for all PBDE congeners. The GC-MS sensitivity was calculated as the average plus three times the standard deviation of peak area when pesticide grade acetone was directly injected into the GC. The GC-MS sensitivity was approximately in the same range as the MDL (three times lower) for all congeners analysed, confirming that the entire analytical procedure is free from contamination or interferences.

For BDE-47, the secondary ions *m/z* = 486 and 484 represented 84%±5% and 55±6% of the quantification ion *m/z*=326, respectively. For penta-BDEs (99 and 100), the secondary ions *m/z* = 404 and 408 represented 99%±10% and 33±13% of the quantification ion *m/z*=406, respectively. Average sample secondary ion ratios were between 1 and 6% different from the ratio obtained for standards, i.e. substantially lower than the 20% variation recommended by USEPA Method 1668a (1999) for PCB congener analysis in marine biota tissues.

IV – 3 – 6 PBDE recovery in spiked tissues

The accuracy of the method was evaluated by checking the recovery of spiked BDE-47, 99 and 100 at five concentrations in the four tissue types (See Figure IV-4). The measured concentration for all congeners was linear with respect to spiked concentrations in all tissues

(r^2>0.90). Average analyte recovery was higher for salmon muscle (94%±7%) and seabass liver tissues (97%±14%) than for conger eel muscle (89%±14%) and green mussel tissues (89%±7%). Average recovery was slightly higher for congeners 99 (92%±11%) and 100 (95%±14%) which are penta-brominated, than for the congener 47 (89%±8%) which is tetra-brominated.

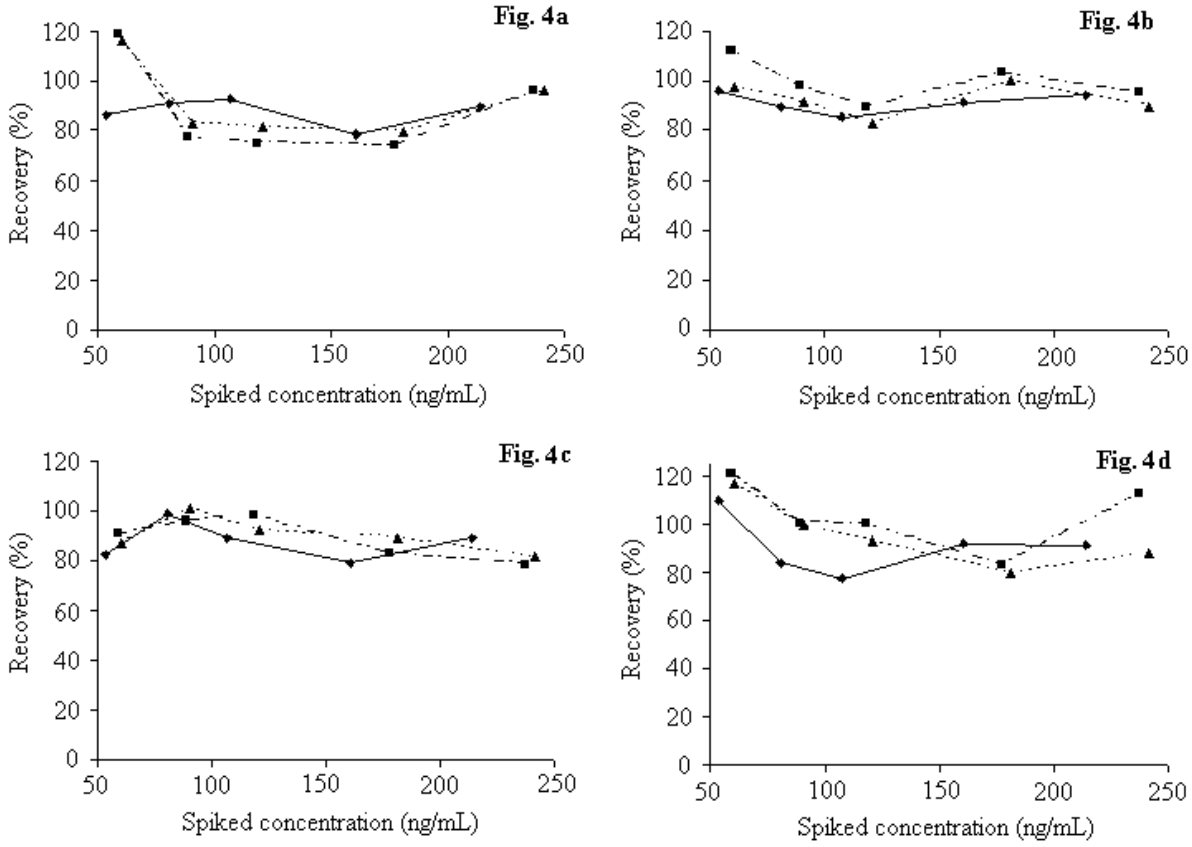

Figure IV-4: Individual recoveries of BDE-47 (circles), 99 (squares) and 100 (triangles) spiked at various levels in conger eel muscle tissues (Fig 4a), salmon muscle tissues (Fig 4b), green mussel soft tissues (Fig 4c) and seabass liver tissues (Fig 4d).

IV – 3 – 7 Analytical precision

The relative standard deviation (RSD) was evaluated at the level corresponding to the medium value of the calibration range (i.e. approximately 100 ng/mL in the final extract). RSD was calculated for seven samples i.e. three fish muscle tissues, two fish liver tissues and

two mussel tissues. The RSDs for the seven replicate analyses were 6.8%, 13.2% and 11.9% respectively for BDE-47, 99 and 100.

IV – 3 – 8 Comparison of MAE and Soxhlet extraction for PBDEs

SRM 2978 and SRM 1588a were both analyzed using the MAE and Soxhlet extraction procedures. The average recovery for PCBs and organochlorine pesticides was higher than 80% for both SRM 2978 (See Section IV-3-1) and SRM 1588a (See Table IV-3).

Analyte	**MAE**	**Soxhlet**
alpha-HCH	107	102
gamma-HCH	95	58
cis-chlordane	95	98
p,p'-DDE	88	86
p,p'-DDD	149	151
p,p'-DDT	107	128
heptachlor epoxide	77	43
PCBs[a]	79	84
Average recovery ± SD	83%±22%	88%±25%

Table IV-3: Comparative recoveries of OCPs and PCBs for MAE and Soxhlet extraction of SRM 1588a. [a] average value for PCB congeners 18, 22, 28, 31, 44, 49, 52, 66, 70, 74, 95, 105, 110, 118, 138, 149, 151, 153, 156, 170, 180, 183, 187, 189, 194.

Concentrations of BDE-47, 99 and 100 were measured in the two SRMs using MAE and Soxhlet extraction (See Table IV-4). Both extraction methods yielded similar results, with MAE yielding slightly lower concentrations than Soxhlet extraction (less than 15% variation). BDE-47 was the dominant congener in the two SRMs analysed, reaching 24 ng/g dw in SRM 2978 and 21 ng/g ww in SRM 1588a.

	SRM 2978		SRM 1588a	
Analyte	**MAE**	**Soxhlet**	**MAE**	**Soxhlet**
BDE-47	24.4	24.6	20.9	21.0
BDE-99	5.4	6.3	2.6	3.0
BDE-100	3.3	3.9	1.6	1.9

Table IV-4: Concentration (ng/g) of BDE-47, 99 and 100 in SRM2978 (dw) and SRM 1588a (ww) determined using MAE and Soxhlet extraction.

IV – 3 – 9 Gravimetric determination of tissue lipid content

The lipid content obtained gravimetrically following MAE was plotted versus the lipid content obtained following Soxhlet extraction (See Figure IV-5).

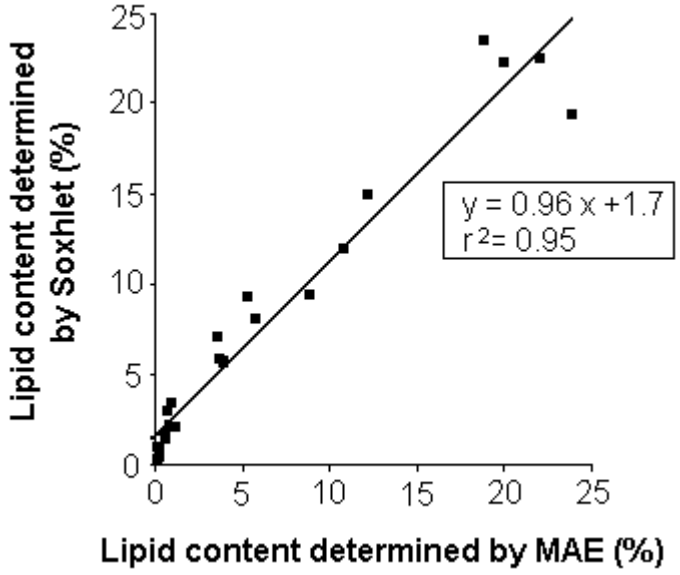

Figure IV-5: Comparative lipid content determined gravimetrically using Soxhlet extraction and MAE for twelve biological tissue types.

The slope of the linear trend line equaled 0.96 with r^2=0.95. However, values obtained with MAE were constantly lower by 1-2% compared to the values obtained with Soxhlet extraction. Therefore, recoveries of MAE versus Soxhlet varied with the initial lipid content (See Figure IV-6). Recovery was >70% for a lipid content of >5%. It is important to note that tissues with the lowest lipid content had also the highest moisture content.

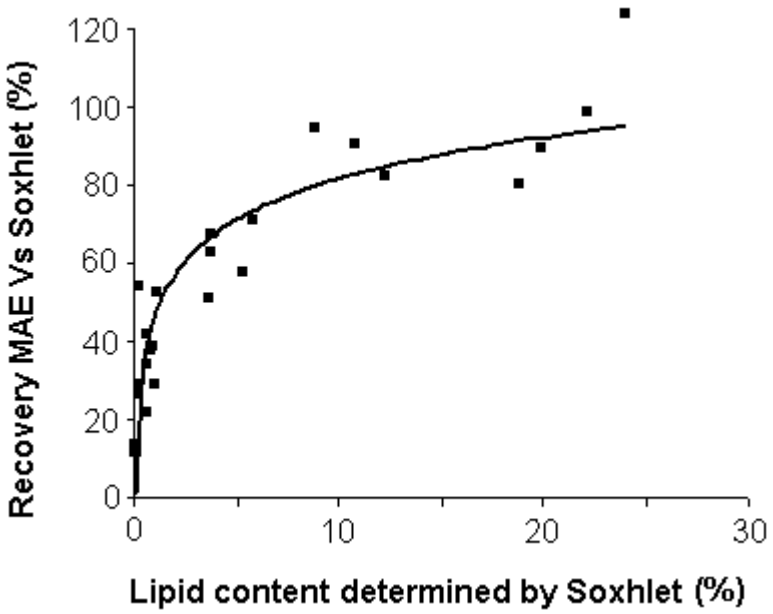

Figure IV-6: Recovery of lipid content using MAE versus Soxhlet extraction as a function of the initial lipid content of the twelve biological tissue types.

IV – 4 Discussion

IV – 4 – 1 MAE performances for POPs extraction

Using the optimized MAE parameters, POPs extraction takes place at a pressure and temperature far above what is conventionally obtained with Soxhlet apparatus, reducing the extraction time considerably. The various recovery tests for PCBs, DDTs, HCHs, chlordanes

and heptachlor, were satisfactory and this confirms the robustness of MAE for the extraction of conventional POPs from marine biota tissues (Jayaraman et al., 2001; Carro et al., 2000). Temperature is a crucial parameter for MAE as it can lead to the degradation or incomplete extraction of analytes (Camel, 2000). However, the set of MAE parameters 115°C/10 bars can be deemed as suitable for PBDE extraction as individual congener recoveries were satisfying. Other extraction parameters applied, including solvent type, extraction time, and microwave power can also be considered as effective for MAE of PBDEs in the tissue matrices analyzed. No specific set of standards are available on the precision/method performance for PBDEs in marine biota tissues. However, the recoveries of surrogate PBDEs are in the range of 70-130%, which is deemed acceptable according to USEPA Method 1688a (1999) for PCB congeners in marine biota tissues. Similarly, the RSD in the present study was comparable with what is reported elsewhere (Christensen et al., 2002) and was substantially lower than a 40% RSD value recommended by USEPA Method 1668a (1999). The MDLs in the present study (<0.05 ng/g ww for all PBDE congeners) are comparable with other studies using conventional extraction techniques (See Table IV-5). The accuracy and precision of the MAE method, combined with the effective removal of matrix interference, makes possible accurate quantitative analysis of PBDE congeners in marine biological tissues. No maximum permissible limit for PBDE in food for human consumption is currently available, but a limit of 2 μg/g for PCBs in fish and shellfish is stipulated by the US Department of Health and Human Services (ATSDR, 2000). If a similar limit were applied to PBDE congeners, then the proposed MAE method is relevant for the rapid, sensitive and quantitative analysis of PBDEs in seafood. The solvent consumption and time requirement of various extraction methods reported recently in the literature for the

extraction of PBDEs from marine biological tissues are presented in Table IV-5. The MAE solvent volume (25 mL) is substantially lower than that required for conventional Soxhlet extraction and column elution, and the extraction time is reduced from several hours to 25 min. An alternative, pressurized liquid extraction technique also reduces time and solvent consumption (Hale et al., 2001), but at a high capital investment cost on equipment (Eskilsson and Björklund, 2000).

Extraction technique	**MDLs (ng/g)**	**Solvent consumption**	**Extraction time**	**Reference**
MAE	0.01 to 0.05[a]	25 mL	25 min	Present study
Soxhlet	0.004 to 0.05[a]	350 mL	6 h	Christensen et al., 2002
Soxhlet	0.01 to 0.2[b]	n.a.	24 h	Akutsu et al., 2001
Column elution	n.a.	300 mL	n.a.	Dodder et al., 2002
Column elution	n.a.	250-350 mL	5-7 min	Alaee et al., 2001
Manual shaking	n.a.	2100 mL	3x10 min	Ikonomou et al., 2001
Pressurized liquid extraction	5[b]	n.a.	2x5 min	Hale et al., 2001
MAE	n.a.	8 mL	7x5.5 min	Vetter, 2001

Table IV-5: Recent reported extraction methods for the determination of PBDEs in marine biological tissues. n.a.:non available data. [a] ww. [b] lw.

IV – 4 – 2 MAE performance for tissue lipid content determination

There were substantial differences in the lipid content measurements of MAE relative to Soxhlet extraction for tissues with lipid content below 5%. For tissue with lipid content greater than 5%, MAE can be used successfully to determine the lipid content.

Numerous methods exist for total lipid determination and results between methods often differ substantially (Honeycutt et al., 1995; Iverson et al. 2001). The only reported use of MAE for lipid determination in the literature gave higher results than manual shaking (Mooibroek, 2002). The difference between MAE and Soxhlet is unclear, but the moisture content may play a decisive role, as the data suggest. The nature of the lipids may also impact the extraction efficiency. For example, lean fish contains mainly membrane-bound phospholipids, whereas fatty fish contain mostly triacylglycerol; and this difference is known to affect lipid content determinations (Boon et al., 2002; Lee et al., 1996). However, the tissue lipid content does not have any substantial effect on the POPs extraction efficiency, as recovery tests yielded satisfactory results in marine biota tissues of both low and high lipid contents. Water in the matrix is known to improve the analyte extraction efficiency with MAE (Eskilsson and Björklund, 2000). POPs extraction may be unaffected as low lipid contents in biological tissues are generally associated with high moisture contents.

IV – 5 Conclusion

The optimized MAE method is a sensitive and robust tool for the analysis of POPs, including PBDEs, in marine biota tissues and has been used extensively in the studies detailed in the subsequent chapters. PCBs, organochlorine pesticides and PBDEs can be analysed simultaneously in a single GC-MS run. Combined with the fast extraction such as MAE, these analytical improvements have enabled the analysis of a large number of samples with a high level of quality assurance.

CHAPTER V – THE GREEN MUSSEL, *PERNA VIRIDIS*, AS A BIOINDICATOR OF POPs IN SINGAPORE

V – 1 Introduction

The Green Mussel, *Perna viridis*, is a mussel species that is naturally prevalent in Asia-Pacific coastal waters (Gosling 1992). As a filter-feeding organism, green mussels have been used as a bioindicator species for various POPs and heavy metals in South-east Asia (Monirith et al., 2000) and America (Rojas de Astudillo et al., 2002), but not in Singapore. Green mussels are naturally occurring in Singapore's coastal waters, and are cultured commercially in the Johore Strait which separates Malaysia from Singapore (Chou and Lee, 1997). Mussels represent the most common species of shellfish cultivated in the world, with more than 1.1 million tons produced in 1998 (Gosling 1992). In 2001, green mussels represented 27% of the volumetric total of seafood generated by the Singapore aquaculture industry (FAO, 2003).

In this study, *P. viridis* was used to investigate the occurrence of POPs in Singapore's coastal marine environment. First, the effect of age and time on the POPs burden in green mussels was investigated in an aquaculture farm in Singapore. Secondly, *P. viridis* samples, collected around Singapore's coast in 2002/2004, were analysed for POPs and heavy metals to evaluate the geographical distribution of contaminants. This represents the first study on the use of *P. viridis* as a bioindicator species for the monitoring of PBDEs in Asia's marine environment. Finally, this study also reports the use of human-cell based bioassay for the

determination of sex hormone activity in extracts of *Perna viridis* sampled from Singapore's coastal waters in 2002. This represents the first measurement of *both* androgenic and estrogenic activities in an environmental biological tissue extract using a human-cell based bioassay. Data on sex hormone activities in *P. viridis* samples collected from the coastal waters of Singapore were then correlated statistically to various parameters measured in the mussels, including contaminant burden, to evaluate the application of the bioassay as an indicator of endocrine disrupting chemicals (EDCs) in biological samples.

V – 2 Materials and Methods

V – 2 – 1 Temporal variation of POPs in *P. viridis*

To study the effect of size and the temporal variation of POPs in cultured *Perna viridis* in Singapore, mussels of different size classes were collected from a local fish farm over a period of 7 months (July 2003-January 2004). The station, located in the East Strait of Johore, near the island of Pulau Ubin (N 01°23.95' E 103°57.85'), was chosen based on the importance of the site for aquaculture in Singapore. Mussels were collected from suspended ropes at constant depths over the duration of the experiment (between 0.5 and 1 m). At each sampling episode, mussels were sorted into distinct size classes (10 mm class sizes) with 2 to 40 individuals (average =17) in each class depending on the availability and the size of individuals and the amount of soft tissues present (See Appendix B). In the laboratory, the length and gender of each specimen were recorded. Gender is easily ascertained for *P. viridis*: female tissues are orange in colour, and male tissues are creamy-white (Gosling, 1992) (the common name refers to the shell colour).

V – 2 – 2 Geographical distribution of POPs in *P. viridis*

Geographical distribution of POPs in *P. viridis* was studied for nine locations around Singapore's coastline in April/May 2002 and 2004 (Figure V-1). The sample in Pulau Tekong (S5), in the northeast, was collected in 2004 only, as the fish farm sample site was established in 2003.

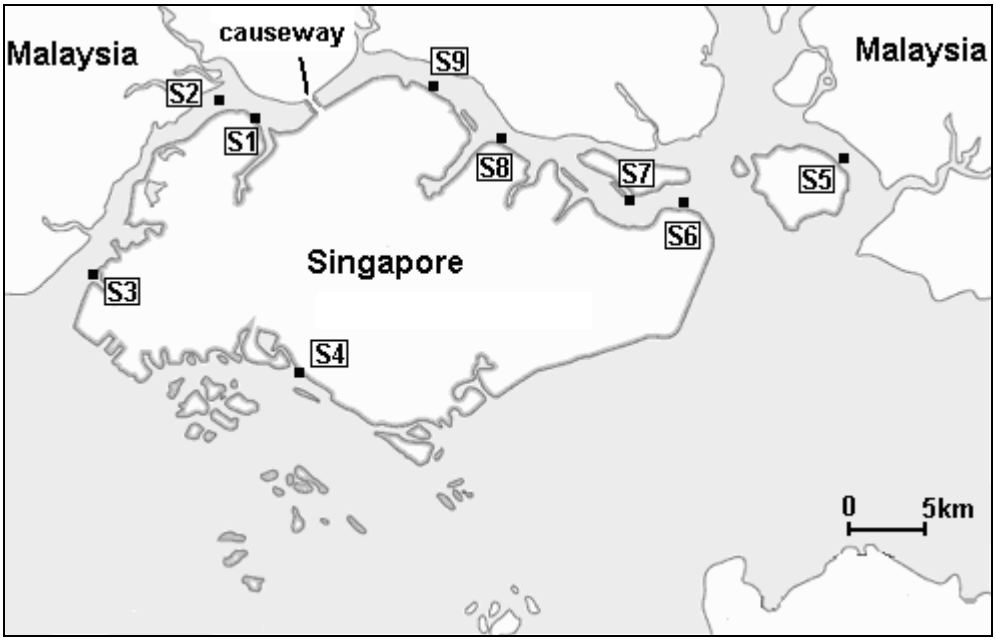

Figure V-1: Sampling locations of *Perna viridis* in Singapore's coastal environment.

The stations were chosen based on their proximity to industrial activities, and their position around the land-link causeway between Singapore and Malaysia in the Straits of Johore. The causeway represents a physical barrier to marine hydrodynamics around Singapore's northern coast. Stations S3, S4 and S9 were selected for their proximity to industrial areas and shipyards. Samples were collected from marine floating structures and shoreline defence walls. One single batch of mussels was collected per site in 2002. In 2004, two batches of mussels were collected per site, when available, to assess variability of POP accumulation between sites. Individuals were collected in the largest and most similar size-range possible

in each location. Twenty to twenty-five individuals were collected from each location, but some were later rejected, as their size was not suitable. In the laboratory, the weight, length and gender of each specimen from all eight locations were recorded (See Table V-1).

Site	Location	Year of collection	Number of individuals	Ratio (male/female)	Average size (cm)	Moisture content (%)
S1	Sungei Buloh	2002	18	0.38	9.7	86
		2004	2 x 25	n.a.	8.4	87
S2	Lim Chu Kang	2002	21	0.62	8.9	79
		2004	2 x 25	0.70	8.1	86
S3	Tuas	2002	20	0.54	10.7	78
		2004	14	n.a.	9.9	89
S4	Jurong	2002	11	0.57	8.4	78
		2004	5	n.a.	9.4	82
S5	Pulau Tekong	2004	2 x 25	n.a.	7.9	87
S6	Changi	2002	20	0.31	9.0	79
		2004	2 x 25	n.a.	6.3	89
S7	Pulau Ubin	2002	20	0.25	10.2	81
		2004	2 x 25	n.a.	4.9	84
S8	Punggol	2002	16	1	9.4	78
		2004	2 x 25	n.a.	8.9	82
S9	Sembawang	2002	20	0.25	9.7	82
		2004	2 x 25	n.a.	8.5	87

Table V-1: Biological characteristics of *Perna viridis* samples collected in Singapore. n.a.: not available.

V – 2 – 3 Sample collection and preparation

Samples were transported in polyethylene bags in ice-boxes to the laboratory for analysis. To prepare samples for POP analysis, soft tissues were removed from the shell and homogenized in a stainless steel blender to form batch samples.

V – 2 – 4 POPs analysis

Except for the monitoring round in 2002, all the mussel samples have been analyzed following the procedure presented in Chapter IV, using MAE extraction. When sample size allowed, samples were duplicated. To further ascertain reproducibility, one batch of mussels from station S6 was analysed four separate times.

Chemical analysis for the monitoring round in 2002 was completed in the laboratories of Pr. Kevin Jones at the Department of Environmental Sciences, Lancaster University, UK. Samples were freeze-dried for transportation to the UK. Approximately, 1 g of freeze-dried *P. viridis* tissue was accurately weighed (+/- 0.1 mg) for analysis, and ^{13}C-labelled PCB congeners 28, 52, 101, 138, 153 and 180 were added as internal standards. The samples were extracted using accelerated soxhlet extraction as described in Section III-4-3, Chapter III. The cleanup and preparation procedure prior to analysis was similar to that presented in Section III-5, Chapter III. PCB and OCP analysis was performed using a HP5890 II gas chromatograph equipped with a HP 5872 S mass selective detector (Helwett-Packard, Palo Alto, CA, USA). Separation was carried out on a CP-Sil8 (Chrompak/Varian, Palo Alto, CA, USA) capillary column (length 50 m, diameter 0.25 mm) using He as the carrier gas. The detector was operated in EI-SIM mode. PCBs 18, 22, 28/31, 44, 49, 52, 54, 70, 87, 90/101,

95, 99, 100, 105, 110, 114, 118, 123, 132, 138, 141, 149, 151, 153, 155, 156, 157, 158, 167, 170, 174, 180, 183, 187, 188, 189, 194, 199 and 203 were quantified in each sample. These congeners were selected due to their toxicity, abundance and persistence in the environment (Jones, 1988). PBDEs were analyzed by gas chromatography using a DB-5ms (J&W Scientific, Palo Alto, CA, USA) capillary column (length 30 m, diameter 0.18 mm) and a quadrupole mass spectrometer (Fisons MD800; Uckfield, U.K.) running in negative chemical ionization (NCI) mode using ammonia as the reagent gas. Helium was used as the carrier gas. Masses 79 and 81 were monitored as they are characteristic of Br. Quantification was performed for PBDEs 17, 28, 32, 35, 37, 47, 49, 66, 71, 75, 77, 85, 99, 100, 119, 138, 153, 154, 166, 181 and 190. The choice of the congeners was based on the composition of common commercial flame-retardants containing pentabrominated PBDE (Sjödin et al., 1998). Quantification of single congeners was performed using a seven-point calibration.

V – 2 – 5 Androgenic and estrogenic activity of mussel extracts

This work is a result of collaboration with the research team of Pr Yong Eu Leong, at the National University Hospital, Singapore. Green mussels, collected for geographical distribution in 2002, were extracted using MAE with 30 mL of methanol / ethanol / DCM / n-hexane / ethyl-acetate mixture (1:1:1:1:1 v/v/v/v/v). The extraction temperature was increased to 110ºC within 10 min and then held for 3 min at this value, using 60% of 1200 W power. Mussel extracts were then screened for activities on androgen receptors (ARs) and estrogen receptors (ERα and ERβ) using a reporter gene bioassay based on a HeLa human cell line, either in the absence, or in the presence of the well-known hormones, androgenic dihydrotestosterone (DHT) or estrogenic 17β-estradiol (E2). The bioassay methodology and

optimization, developed by the Pr Leong's research team, is described in a previous publication (Gong et al., 2003).

V – 3 Results

V – 3 – 1 Method performance and quality control

Quality assurance results and MDLs are summarized in Table V-2 for the various experiments. Recovery standards were satisfactorily recovered in the range of 75 to 125% (mean recovery ranged 80 to 114%). The certified values for standard reference material, i.e. SRM 2978, were achieved, with an average recovery ranging from 89% to 110%.

	Temporal variation experiment	**Geographical distribution, 2002**	**Geographical distribution, 2004**
Surrogates recovery	80%±10%[a]	114%±11%[b]	89%±14%[a]
SRM-2978 recovery	90% ± 23%	110% ± 18%	89% ± 19%
MDL chlordanes [c]	0.02 ng/g ww	0.01 to 0.05 ng/g dw	0.12 ng/g dw
MDL DDTs [c]	0. 1 to 0.4 ng/g ww	0.01 to 0.75 ng/g dw	0.2 to 0.3 ng/g dw
MDL PCBs [c]	0.01 to 0.9 ng/g dw	0.01 to 0.9 ng/g dw	0.02 to 2.0 ng/g dw
MDL PBDEs [c]	0.005 to 0.3 ng/g ww	0.01 to 0.3 ng/g dw	0.1 to 0.3 ng/g dw

Table V-2: Quality assurance results and method limit of detection (MDLs) for the analysis of POPs in *P. viridis* in Singapore. [a] PCB 55 and 61 used as recovery standards. [b] ^{13}C-labelled PCBs used as recovery standards. [c] Range of MDLs for all congeners.

To determine the analytical reproducibility, four replicate analyses were performed on a single green mussel homogenate. Mean RSD for the four replicates ranged from 3.2 to 8.2% according to the analyte, indicating that acceptable homogenisation was achieved. As the

various quality assurance tests yielded satisfactory results, data between the various experiments can be compared. MDLs were slightly lower for the geographical distribution study in 2002 compared to 2004, but are generally comparable between studies.

V – 3 – 2 Size distribution& temporal variation

The levels of DDTs, chlordanes, PCBs and PBDEs in mussel tissues (ng/g ww) versus the shell length between July 2003 and January 2004 are presented in Figure V-2. POPs levels were above the MDLs for all contaminants, except for PBDEs in January 2004. The weak *r* coefficients show no significant relationship between size and POPs levels in the soft tissues.

Average concentrations of POPs in all classes of mussels was compared over time (See Figure V-3). Overall, there was a significant difference between samples over time for all POPs (Kruskal-Wallis, $p<0.05$). Data suggest a slight decrease with time of the POPs loading. Rainfall was highest in August with 407mm Vs <250 mm during the other months (NEA, 2004), but this did not seem to have any influence on the POPs load at this aquaculture site.

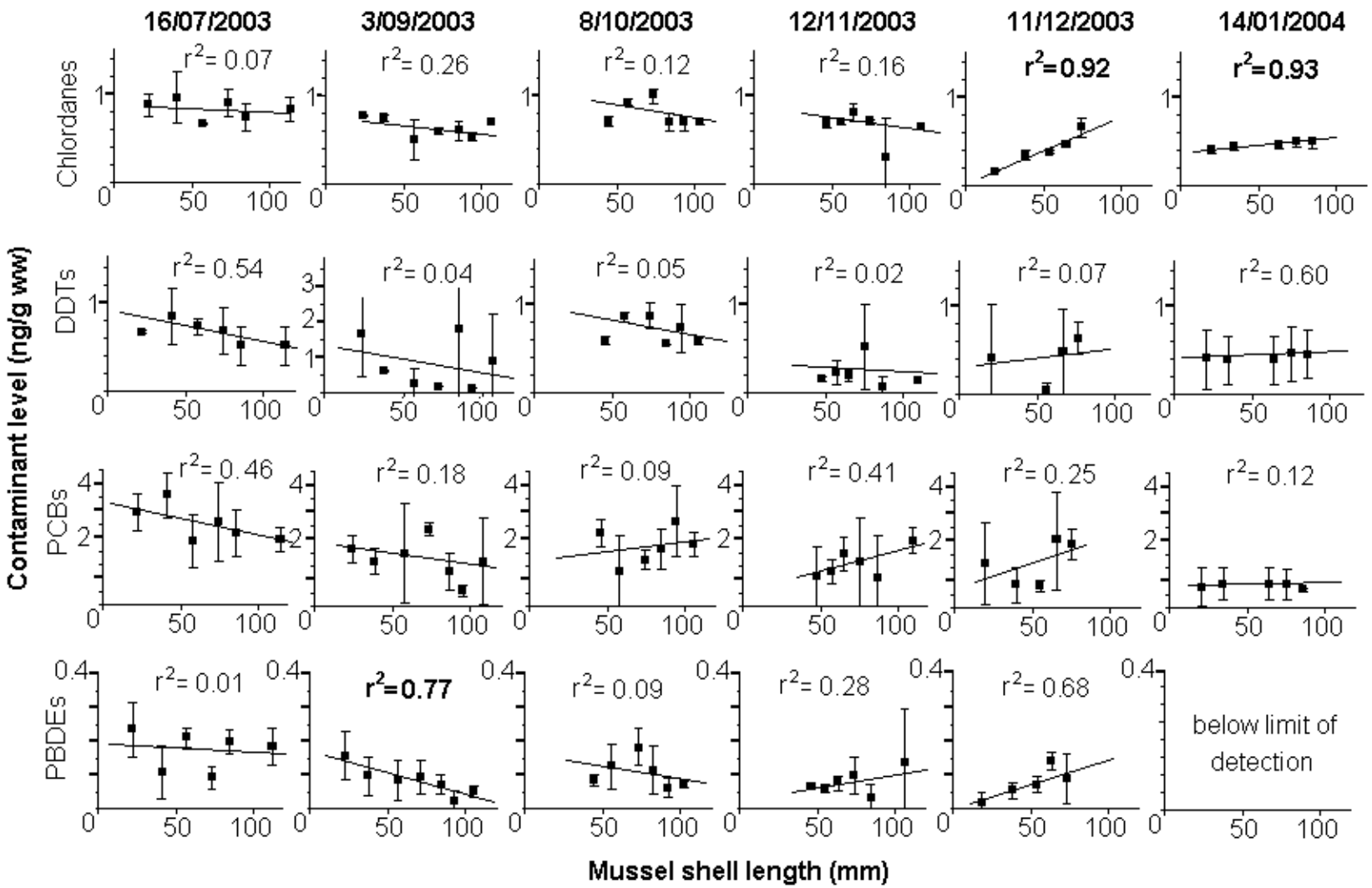

Figure V-2: POPs level (ng/g ww) versus mussel shell length (mm) in *Perna viridis* cultured in Pulau Ubin between July 2003 and January 2004. Figures in bold are significant ($p<0.05$).

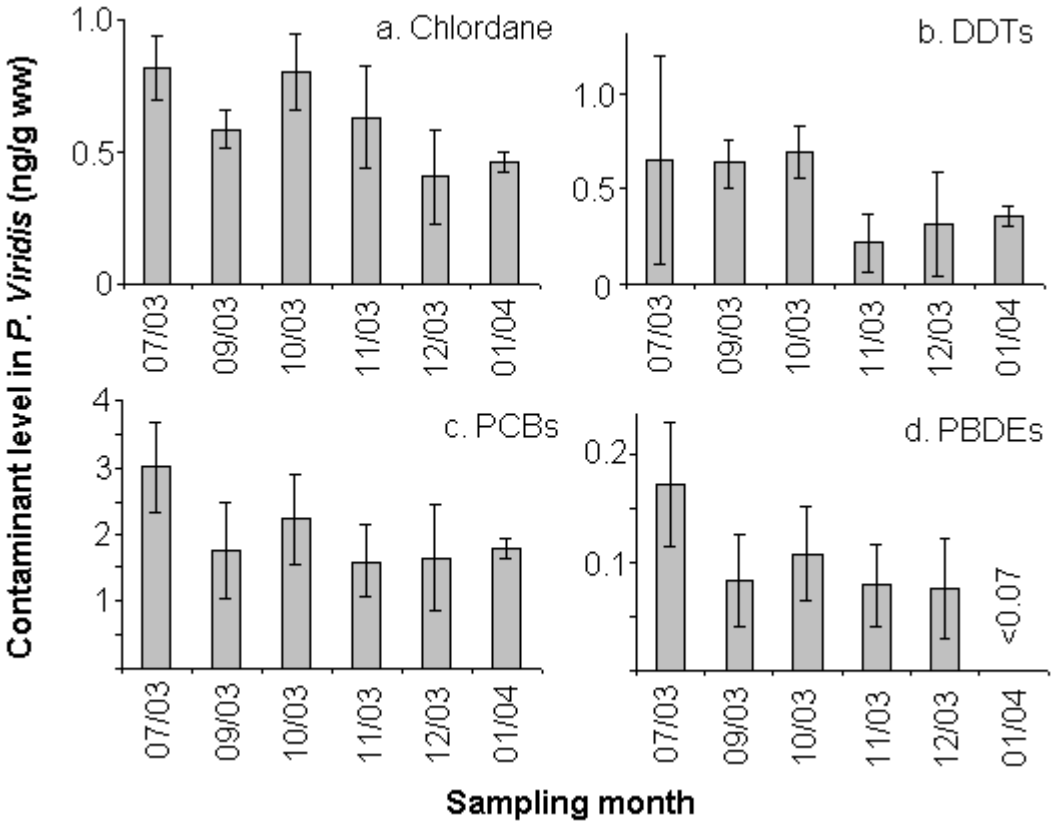

Figure V-3: Average POPs concentration for all size class in *Perna viridis* in Pulau Ubin aquaculture between July 2003 and January 2004 (ng/g ww).

V – 3 – 3 Geographical comparison of POPs in *P. viridis* in Singapore

Concentrations of POPs in *P. viridis* are presented on a dry weight (dw) basis in Table V-3. The levels were below the MDLs for heptachlor (MDL=0.49 ng/g dw), PCNB (MDL=0.93 ng/g dw) and HCB (MDL=0.07 ng/g dw) in 2002 and mirex (MDL=0.07 ng/g dw) in 2004. Concentrations for *p,p*'-DDTs ranged from 2.5 to 54.0 ng/g dw in green mussels sampled in 2002, and from 6.4 to 28.7 ng/g dw in 2004. In 2002, *o,p*'-DDE was quantified, and levels were one or two orders of magnitude lower than the *p,p*'-DDT congeners. Concentrations for chlordanes (α and γ) ranged from 3.1 to 15 ng/g dw in green mussels sampled in 2002, and from 1.9 to 30 ng/g dw in 2004. The highest concentrations for DDTs, chlordanes, mirex and

heptachlor were in mussel samples sampled from stations S3, S4 and S9, which are locations adjacent to ship maintenance yards and an industrial area. Mussels from aquaculture farms (S2, S5 and S7) and the Sungei Buloh National Park (S1) had levels of organochlorine pesticides generally one order of magnitude less than the peak values. In 2002, relationships were investigated between POPs levels and biological parameters (sex ratio, lipid content, moisture content, shell size), but no significant correlations were found ($r^2<0.15$).

Different PCB congener suites in green mussel tissues were analyzed in 2002 and 2004. However, as the congeners, common to both monitoring rounds, represent between 92% and 100% of the total PCB load, data can be considered comparable between the two monitoring rounds. Concentrations for PCBs ranged from 6.1 to 82 ng/g dw in green mussels sampled in 2002, and from BLD to 10.8 ng/g dw in 2004. The highest concentrations of PCBs, in both sampling years, were found at station S9, i.e. near ship maintenance yards. Mussel samples from stations S3 and S4, which are also near ship maintenance yards and an industrial area, also had elevated PCB concentrations.

In 2002, all 21 PBDE congeners were identified in the mussel tissues. The sum of congeners 47, 99, 100, 153 and 154 represented 70% to 93% of the total PBDE load in 2002 and were therefore selected for analysis in the 2004 monitoring round. Concentrations of PBDEs ranged from 2.5 to 54.0 ng/g dw in green mussels sampled in 2002, and from 6.4 to 28.7 ng/g dw in 2004. In most of the samples, BDE-47 was the dominant congener, followed by BDE-99 and BDE-100.

		p,p'-DDTs	*o,p*'-DDE	γ-chlordane	α-chlordane	heptachlor	mirex	PCBs	PBDEs
S1	2002	13.9	0.75	1.3	1.8	<0.49	0.31	17 [a]	2.1 (1.4) [c]
	2004	8.5±3.0	n.a.	2.7±0.4	2.2±0.2	0.4±0.2	<0.07	1.0±0.8 [b]	0.4±0.2 [d]
S2	2002	8.2	0.32	1.5	2.2	<0.49	0.28	23 [a]	2.3 (1.8) [c]
	2004	6.4±2.1	n.a.	3.9±0.9	3.1±0.8	0.2±0.3	<0.07	3.1±0.1 [b]	0.7±0.1 [d]
S3	2002	2.5	0.13	2.8	3.2	<0.49	0.26	6.1 [a]	38 (34) [c]
	2004	28.7±25.7	n.a.	17.4±11.7	12.2±8.4	0.8±0.1	<0.07	10.4±14.4 [b]	10.4±4.1 [d]
S4	2002	54.0	0.16	7.9	7.2	<0.49	1.5	44 [a]	13 (10) [c]
	2004	19.6±12.5	n.a.	6.5±4.8	4.8±3.6	0.2±0.0	<0.07	3.1±4.4 [b]	1.9±1.2 [d]
S5	2004	7.2*	n.a.	1.0*	0.9*	0.3*	<0.07	BLD	0.1 *[d]
S6	2002	19.8	0.50	2.3	2.8	<0.49	0.52	15 [a]	3.3 (2.6) [c]
	2004	8.1*	n.a.	3.0*	2.9*	0.8*	<0.07	0.5* [b]	1.5*[d]
S7	2002	5.1	0.14	1.8	2.6	<0.49	0.30	15 [a]	2.1 (1.6) [c]
	2004	9.3±0.2	n.a.	3.4±0.0	3.2±0.0	0.6±0.0	<0.07	1.5* [b]	0.5±0.1 [d]
S8	2002	8.3	0.20	2.3	3.3	<0.49	0.36	18 [a]	5.3 (4.5) [c]
	2004	7.3*	n.a.	7.0*	6.2*	0.9*	<0.07	0.9±0.1 [b]	4.3*[d]
S9	2002	21.1	0.40	4.2	5.7	<0.49	0.60	82 [a]	4.7 (3.7) [c]
	2004	13.0±3.7	n.a.	16.3±3.1	12.4±2.3	7.8*	BLD	10.8±3.7 [b]	2.8±0.2 [d]

Table V-3: Concentrations of POPs in green mussel samples collected in Singapore's coastal area (μg/g dry weight).* some replicate values were BLD (below limit of detection). [a] Sum of PCB congeners listed in paragraph V-2-4.[b] Sum of PCB congeners 17, 18, 28/31, 33, 44, 49, 52, 70, 74, 82, 87, 90/101, 95, 99, 105, 110, 118, 128, 132, 138, 149, 151, 153, 156, 169, 170, 171, 177, 180, 183, 187, 194, 199, 201, 205, 206, 208 and 209. [c] Sum of PBDE congeners listed in paragraph V-2-4. The sum of congeners 47, 99, 100, 153 and 154 is presented between brackets.[d] Sum of PBDE congeners 47, 99, 100, 153 and 154. n.a.: not available.

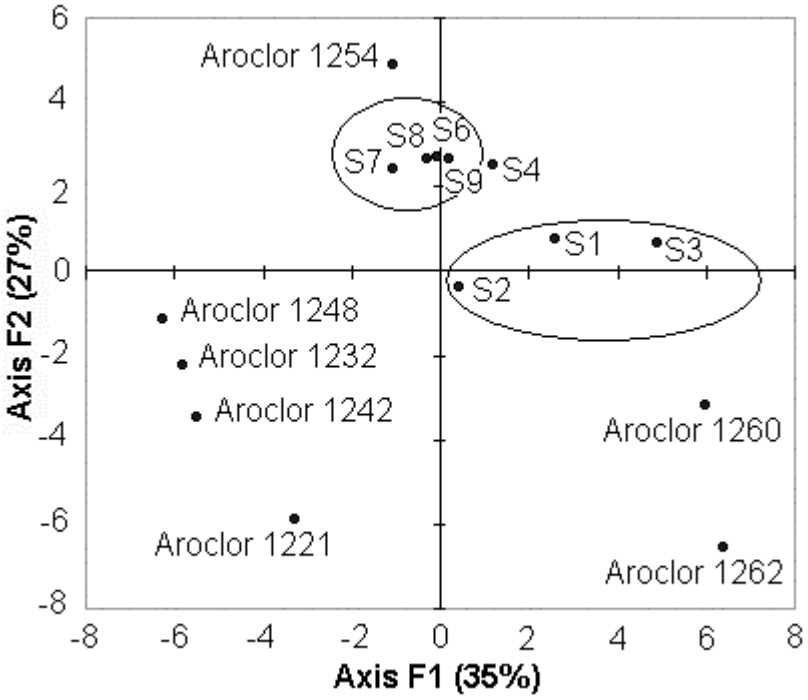

Figure V-4: Biplots of the first two principal components of relative individual PCB congener profile in *Perna viridis* in 2002 and in Aroclor mixtures 1221, 1232, 1242, 1248, 1254, 1260 and 1262.

Principal component analysis (PCA) was performed to compare the relative PCB congener profile of mussel tissues analysed in 2002 and the commercial Aroclor mixtures (See Figure V-4). Mussels analysed in 2004 could not be used in the PCA as a number of congeners were below the limit of detection, and therefore not suitable for statistical calculations. The first principal component, F1, has a significant positive loading on hexa-CBs, hepta-CBs and octa-CBs and a significant negative loading on tri-CBs and tetra-CBs. The second principal component, F2, has a significant positive loading on tetra-CBs, penta-CBs and hexa-CBs and significant negative loading on tri-CBs, hepta-CBs and octa-CBs. Penta-CBs and hexa-CBs are the major PCB groups typically found in *P. viridis*. Thus, mussel samples appear in the upper right-hand side of the biplot in Figure V-4. The closest match in the PCB data for

P.viridis samples collected in this study is Aroclor 1254. The slight discrepancy is due to the presence of PCB-149 in mussel tissue and a greater prominence of PCB 110 and 118 in Aroclor 1254. PCA analysis revealed that samples from the west Straits of Johore (S1, S2 and S3) contain more penta-CBs and less hexa-CBs than samples from the east Straits (S6, S7, S8 and S9). The sample collected in the south of Singapore (S4) has an intermediary pattern of PCB contamination.

Similarly, PCA was performed on the relative concentrations of major PBDE congeners 47, 99 and 100 in *P.viridis* tissues in 2002, and in the commercial mixtures Bromkal 70-5DE and DE-71 (See Figure V-5). Mussels analysed in 2004 could not be used in the PCA as a number of congeners were below the limit of detection, and therefore not suitable for statistical calculations. The sample score derived from data in mussels in Denmark (Christensen and Platz, 2001) and the Netherlands (De Boer and Cofino, 2002) have been added for comparison. Samples collected in all locations (except S3 and S8) are similar in composition to Bromkal 70-5DE. Amongst this group, samples from the west Strait of Johore differ slightly from ones from the east, with an intermediary composition for sample S4. In sample locations S3 and S8, the congener composition is closer to the commercial mixture DE-71. The pattern for relative concentrations of major PBDE congeners 47, 99 and 100 in mussels do not present obvious similarities with data from Netherlands and Denmark.

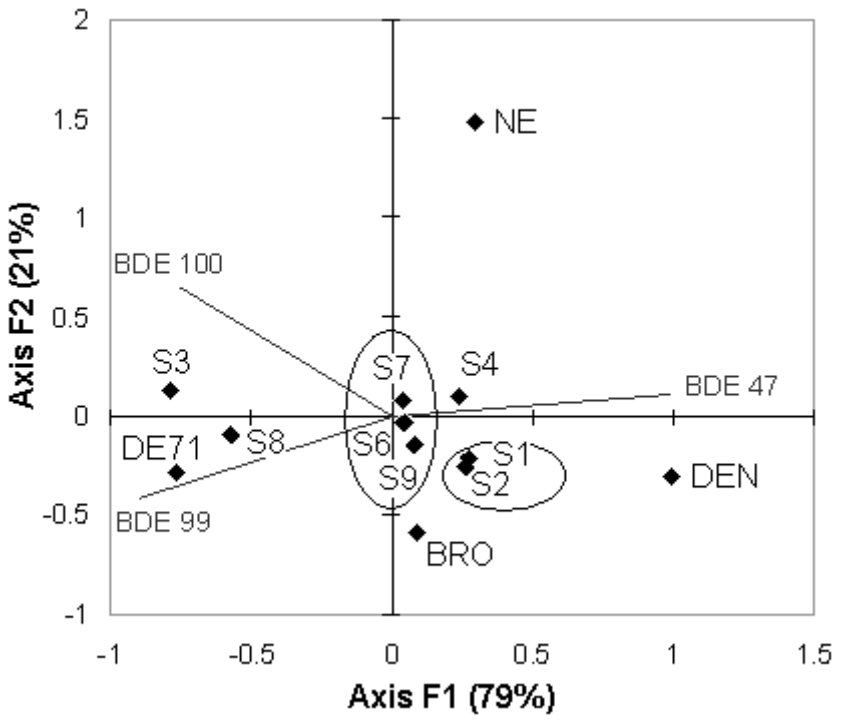

Figure V-5: Biplots of the two first principal components comparing relative ratio of PBDE congeners 47, 99 and 100 in *Perna viridis* from Singapore in 2002, in pentabrominated commercial mixtures Bromkal 70-5DE (BRO) and DE-71, and in mussels analysed in Denmark (DEN) (Christensen and Platz, 2001) and in the Netherlands (NE) (De Boer and Cofino, 2002).

V – 3 – 4 Sex hormone activities of mussel extracts

Sex hormone activities of *Perna viridis* extracts are presented in Figure V-6. AR activities in the *P. viridis* extract in the absence of the DHT 0.1 nM were comparably low between sample locations (<1% of DHT 0.1 nM). In contrast, AR activity in the *P. viridis* extract in the presence of the DHT 0.1 nM ranged from 112% to 340% of the DHT alone, thereby indicating a strong increase in hormone activity in the presence of androgens. Differences in AR activity in the presence of 0.1 nM DHT were significant between sample locations (Kruskal-Wallis, $p<0.05$). The strongest effects were found in *P. viridis* samples taken from stations S3, S4 and S9 (See Figure V-1).

ERα activity in the *P. viridis* extract, in the absence of E2 10 nM, reached 49.6% of the activity of E2 10 nM in station S1, and was generally constant in samples from all other locations (18.3%±2.2% of E2 10 nM). The E2 10 nM estrogenic reference hormone displayed higher ERα activity in the presence of the *P. viridis* extracts for all locations except S2 and S7, ranging 98.1% to 216.9% of the activity observed for E2 alone. Differences in ERα activity, in the presence of 1 nM E2, were significant between each sample site (Kruskal-Wallis, $p<0.05$). The greatest increase in ERα activity was observed for samples taken from stations S3 and S4.

ERβ activity in the *P. viridis* extract, in the absence of E2 10 nM, was more variable than ERα activity, where peak values were found in samples taken from station S1 (31.3% of E2 10 nM) and S4 (16.0% of E2 10 nM). The ERβ activity of E2 10 nM in the presence of the *P. viridis* extract ranged 54.9% to 115.4% of the E2 10 nM alone. ERβ activity in the presence of 1nM E2, in samples S1, S2, S8 and S9 were significantly lower than the activity of the reference hormone E2 alone (Kruskal-Wallis, $p<0.05$), and therefore inhibited the ERβ activity of E2. The highest increase in ERβ activity was observed for samples from station S3 and S4 and S7.

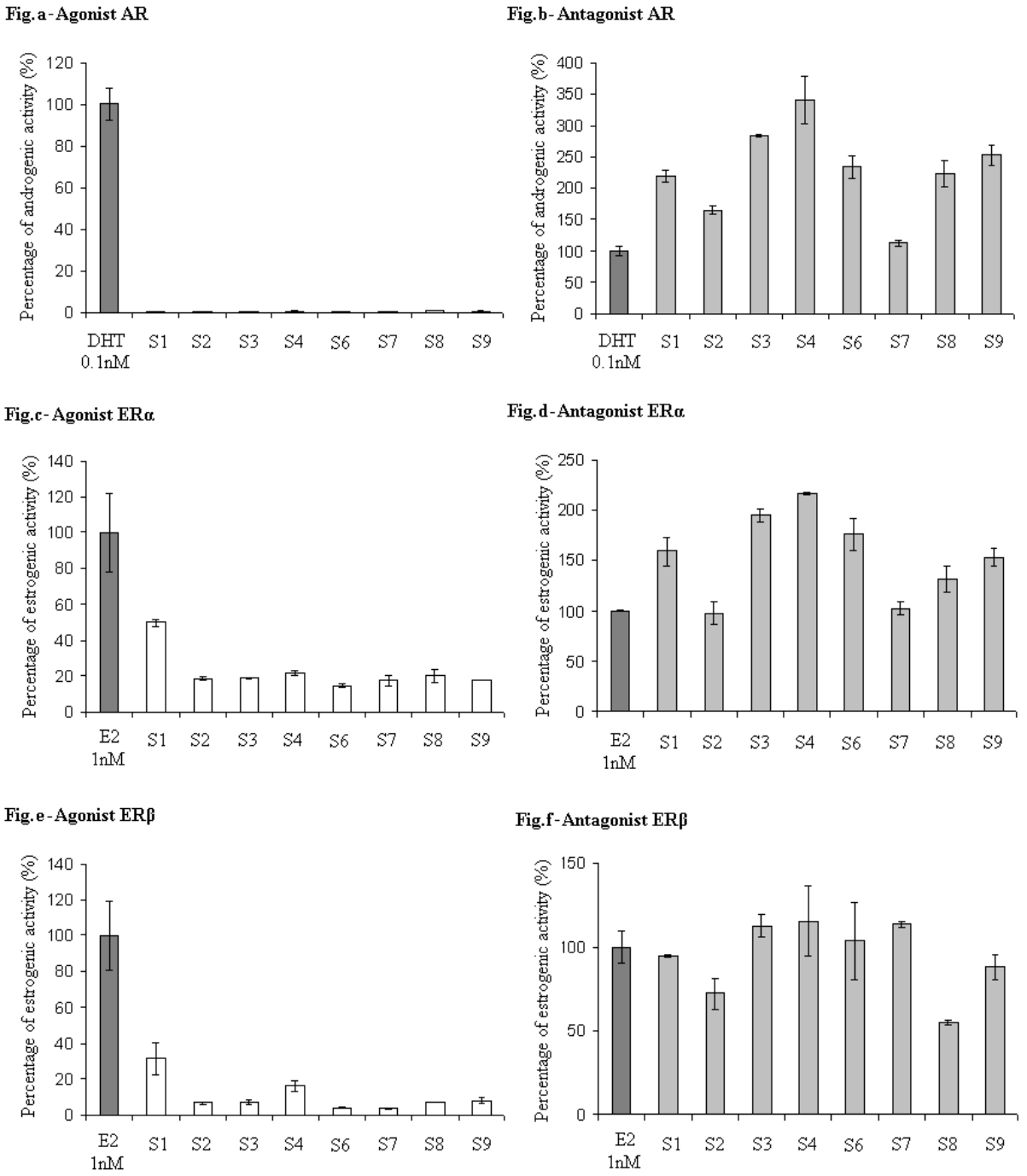

Figure V-6: Sex hormone activities of extracts of *P. viridis* (Average ± SD) as a percentage of the reference hormone. Figures a, c and e represent the activities of the mussel extracts alone. Figures b, d and f represent the mussel extracts in the presence of the reference hormone.

As shown in Figure V-6, AR activity in the presence of 0.1 nM DHT is the sex hormone activity with the greatest variability in *P. viridis* tissues between sample locations i.e 112.3%-340.1% of DHT 0.1 nM. AR activity in the presence of 0.1 nM DHT has a significant (p<0.05) and positive correlation with the sum of α- and γ-chlordane levels (r=0.759), as well as the total concentration of POPs (r=0.725) – see Figures V-7.

ERα activity in the presence of 1 nM E2 shows similar trends, although the r coefficient is weaker and not significant i.e r=0.582 with total concentration of POPs. In contrast, activities of the mussel samples in the presence of reference hormones do not show any strong linear correlation with any heavy metal or biological parameters of the mussels (i.e. specimen size, moisture and lipid content, and batch sample sex ratio). Activities of samples, in the absence of the reference hormone, do not show any strong proportional correlations with either heavy metal or POP tissue concentrations.

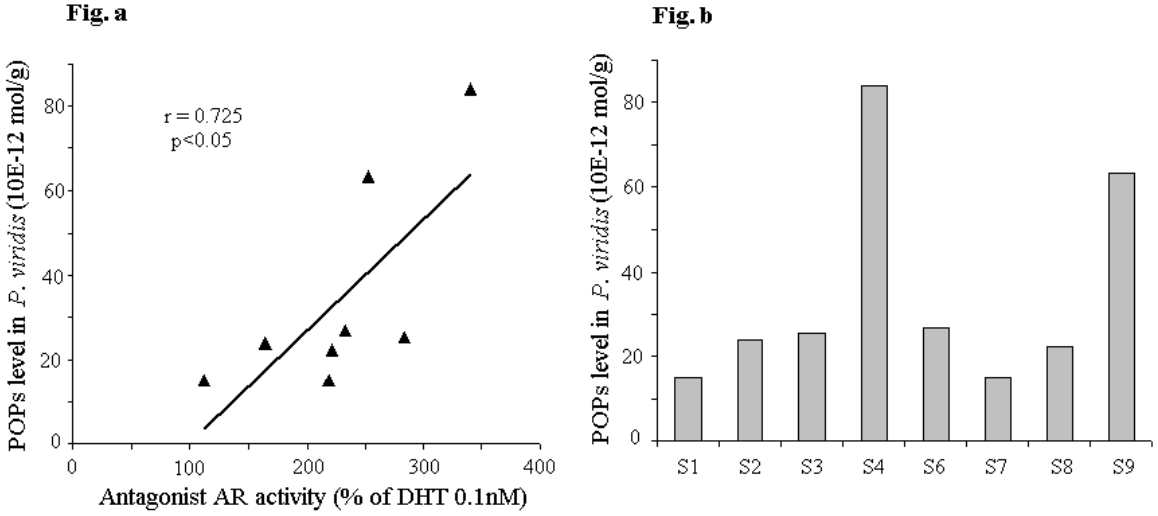

Figure V-7: Relationship between AR activity in presence of DHT and total levels of POPs (a) in the *P. viridis* tissues. Total levels of POPs, in mol/g ww, in the green mussel tissues collected around Singapore are presented in Figure b.

V – 4 Discussion

V – 4 – 1 *P. viridis* as a bioindicator

Some studies have reported an occasional effect of size or tissue weight on the pollutant load in mussels (Phillips, 1985). In the present study, no clear effect of the size of the green mussel on the levels of POPs, including PBDEs, in the soft tissues was apparent from this study. It is important to note that, because of a few cases (e.g. chlordanes on the 11/12/2003), it is a better practice to use organisms of a similar size when performing geographical distribution studies of contaminants. As well as shell size, there was no significant relationship between contaminant levels and other biological parameters of the mussels, such sex ratio and lipid content, which is an important criterion for the choice of a bioindicator (Kaiser, 2001). Phillips (1985) also reported that sex ratio was an unimportant parameter influencing the contaminant concentrations in *P. viridis*. The present data support the use of *P. viridis* as a bioindicator for POPs, including PBDEs.

V – 4 – 2 Geographical distribution of POPs in *P. viridis* in Singapore

Contamination of mussel tissues with PCBs and organochlorine pesticides is present in areas where industrial and shipping activities are intense. In a follow-up study of the present work, Wurl and Obbard (2004b) also noted that levels of PCBs in marine sediments were higher in samples collected near industrial or shipyards areas in Singapore. Levels of POPs in *P. viridis* from all sample locations except S3, S4 and S9 correspond to the lower range of values reported in *P. viridis* tissues from elsewhere in Asia and America (See Section II-3-4, Chapter II). PCBs and organochlorine pesticides levels reported for stations S3, S4 and S9

are in the range of values for green mussel tissues from the marine environment of Hong Kong, which is regarded as heavily contaminated (Richardson et al., 2001).

The highest concentration of PBDEs was found in tissue samples from Station S3. This sample station is close to the industrial area of Tuas, where a number of electronic manufacturing and electronic waste recycling companies are present. PBDEs are commonly incorporated into polymers in electronic components (De Wit, 2002; Jakobsson et al., 2002). The observed levels may be due to the discharge of effluents derived from the material used in the production or the dismantling of electronic equipment. Mussel samples from Station S4, which is adjacent to an industrialized area and shipping lane in the south of Singapore, also had elevated PBDE concentrations.

No comparative PBDE data are available for *P. viridis* outside Singapore, although PBDE levels have been previously reported in blue mussel (*Mytilus edulis*) tissues taken from marine waters off Denmark, Greenland and Netherlands (Christensen and Platz, 2001; Christensen et al., 2002; De Boer and Cofino, 2002). Concentrations of the PBDEs 47, 99, 100 and 153 in *M. edulis* tissues ranged from 0.080 to 0.81 $ng.g^{-1}$ ww in Denmark, 0.11 $ng.g^{-1}$ ww in Greenland and 1.3 $ng.g^{-1}$ ww in Netherlands. On a wet weight basis *P. viridis* tissues from Singapore ranged from 0.29 to 8.6 and from 0.01 to 1.1 $ng.g^{-1}$ ww in 2002 and 2004 respectively. Therefore, in comparative terms, PBDE levels in mussel tissues from Singapore are up to an order of magnitude greater than available data from elsewhere. PBDE levels found in this study are in the range of concentrations reported in a variety of fish and

marine mammals in Europe and America (De Wit, 2002) and higher than in tuna sampled from around the world (Ueno et al., 2004).

V – 4 – 3 History of POPs in *P. viridis* in Singapore

In the aquaculture site of Pulau Ubin (S7), measured levels of POPS decreased over a 6 month period. This result concurs with the slight decrease in levels found in green mussels collected in Singapore in 2004 relative to 2002 for PCBs, DDTs and PBDEs, but not chlordanes. This trend might reflect a decreasing occurrence of these POPs in Singapore's marine environment. It is important to note that the ban of chlordanes in Singapore is quite recent (1999) and that chlordanes were still used in Malaysia until recently (Lee, 2002).

In all samples analysed in this study, the levels of *p,p'*-DDT were found to be lower than the DDT metabolites *p,p'*-DDE and *p,p'*-DDD. As the commercial DDT mixture is principally composed of *p,p'*-DDT, it can be assumed that the mussels are no longer exposed to this pesticide, where metabolisation of bioaccumulated DDT has occurred.

The PCB congener profile in mussel tissues best matched the commercial mixture Aroclor 1254, one of the main components of Askarel, the common PCB-containing product previously used in transformers and capacitors (Erikson, 1992). A similar match has also been observed in marine crabs and fishes in Hawaii (Miao et al., 2001). As the levels are considered to be low, PCBs in mussels are likely to be a result of the historical use of Askarel in Singapore.

BDE-99 and BDE-47 are the two main components of the commercial pentabrominated diphenyl ethers mixtures, including Bromkal 70-5DE, which has a composition of 35% and 37% respectively (Sjödin et al., 1998) and DE-71, with a composition of 47% and 25% respectively (Dodder et al., 2002). These two PBDE congeners were dominant in the *P. viridis* tissues, with a lower concentration of BDE-100. In most sample locations (except S3 and S8), BDE-47 was present at higher tissues concentrations than BDE-99. The uptake of BDE-47 and BDE-99 in *M. edulis* is virtually identical, but the depuration rate of BDE-99 is faster (Gustafsson et al., 1999). As a consequence, an organism exposed to penta-PBDE contamination, can be expected to have a higher tissue concentration of BDE-99 than BDE-47. When the organisms are no longer exposed, BDE-99 is likely to be excreted with BDE-47 then becoming the dominant congener. In samples collected from Tuas (Station S3) and Punggol (Station S8), BDE-99 was more prevalent in 2002 and equal to BDE-47 in 2002, which indicates a relatively recent contamination event. Amongst numerous studies summarized in a recent review by De Wit (2002), no reference reported this ratio for BDE-99 and BDE-47 in a range of marine tissue types.

V – 4 – 4 Sex hormone activities of mussel extracts

Peaks of AR or ER activity in mussel extracts in the presence of the reference hormone corresponded to the sites where peak of POPs contamination. Pearson correlation analysis shows that total POPs has a positive and significant correlation ($p<0.05$) with the pattern of the AR activity of the *P. viridis* extracts in the presence of 0.1 nM DHT. In contrast, no significant correlation was apparent for sex hormone activities of the mussel extracts, in the presence of reference hormones, and heavy metal concentrations, or any measured biological

parameter. This information suggests a relationship between the presence of POPs in the mussel extracts and the androgenic activity of the bioassay (See Figure V-6). Sex hormone activities in reporter gene assays using human cell lines have been previously assayed for POPs, including chlordanes (Legler et al., 1999), DDT (Legler et al., 1999; Maness et al., 1998) and PCBs (Schrader and Cooke, 2003). Endocrine disruption has also been demonstrated for mirex in mice (Dai et al., 2001), and intimated for PBDEs in a study on seals (Hall et al., 2003).

The sum of total POP concentrations in wet mussel tissue ranged from 14.10^{-12} to 84.10^{-12} mol per gram. After extraction and dilution, these concentrations correspond to 0.023 nM to 0.140 nM used in the bioassay, which are well below threshold concentrations observed for single contaminants previously reported (Legler et al., 1999; Schrader and Cooke, 2002). Still, mixtures of individual EDCs are known to induce synergistic responses in endocrine bioassays when present at levels below their individual threshold concentrations (Silva et al., 2002). However, the association between the bioassay and specific congener should be considered carefully. First, the effects of single chemicals are very complex and even a single PCB congener, for example, can exhibit both estrogenic and anti-estrogenic effects (Gregoraszczuk et al., 2003). Additionally, the extraction technique is designed for monitoring the summation effects of all potential EDCs present in green mussel samples. Other chemicals, including dioxins, alkyl phenols, phthalate esters, Toxaphene, contaminant metabolites, estrogenic drugs and steroids are all known EDCs (Sonnenschein and Soto, 1998) and are likely to be extracted with the solvent mixture (Camel, 2000; Schmidt and Steinhart, 2002). *In vitro* activity on HeLa cell based assays are known to be responsive to

chemicals such as phthalate esters (Zacharewski et al., 1998) and hydroxylated PCBs (Moore et al., 1997), and many xenobiotic compounds are known to have synergistic endocrine effects (Kortenkamp and Altenburger, 1998). The presence of other EDCs in the mussel tissues such as dioxins, alkyl phenols or phthalate esters may therefore account for the remaining variability observed for endocrine activity of the mussel extracts. Therefore, the bioassay should be regarded as a tool to monitor cumulative effects of all potential EDCs in the mussel tissue extracts to provide a more holistic measure of the impact of complex multi-chemical mixtures on marine biota.

V – 5 Conclusion

The experiments have shown that green mussel, *P. viridis* can be used successfully as a bioindicator for POPs, including PBDEs, in Singapore. The combination of the use of green mussels and the human-cell based bioassay also offers a new understanding of the presence and potential impacts of endocrine disrupting chemicals on marine biota.

Although consumption of penta-BDE commercial mixtures in Asia has been reported to be negligible in 1999 (De Wit, 2002), this study indicates that contamination of biota by penta-BDE mixtures is prevalent in Singapore. As mussels are low in the food chain, elevated concentrations of PCBs and PBDEs may be expected in organisms at higher trophic levels in Singapore's marine food webs.

CHAPTER VI – POPs IN MANGROVE FOOD WEBS OF SINGAPORE

VI – 1 Introduction

Mangroves are an important, yet endangered tropical ecosystem in South-east Asia. Mangroves covered an estimated 13% of Singapore's land in 1820's to less than 0.5% today (Ng and Sivasothi, 1999). They are unique ecosystems with a large biodiversity, and new species continue to be recorded in local mangrove (Ng et Sivasothi, 1999). Mangroves also have an important socio-economic role in local aquaculture and serve as nursing grounds for coastal fish stocks (Mumby et al., 2003). They also provide a wide range of food organisms that are commonly consumed by humans in South-east Asia (e.g. mussels, rodong shell, crab, mullet and even monitor lizard) and aquaculture products (prawns, fish).

The biomagnification of POPs has been well documented for freshwater ecosystems (e.g. Stapleton et al., 2001), and for marine food webs in Polar Regions (e.g. Dietz et al., 2000) and temperate regions (e.g. Law et al., 2003; Voorspoels et al., 2003). However, the presence of POPs in tropical mangrove ecosystems is restricted to just a few studies in Mexico (Páez-Osuna et al., 2002) and Hong Kong (Liang et al., 1999; Zheng et al., 2000), with limited information available on levels in biota. This chapter presents data on the levels of POPs determined in seawater, sediments and twenty-four biota species at two mangrove sites in Singapore. This is the first such data reported for mangrove ecosystems in Singapore.

VI – 2 Materials and Methods

VI – 2 – 1 Study area

For the purposes of this study, organisms were collected within a week period in April 2004 at two mangrove sites: Sungei Buloh and Sungei Khatib Bongsu (See Figure VI-1). These sites are located on each side of the land-link causeway between Singapore and Malaysia in the Straits of Johore. The causeway represents a physical barrier to marine hydrodynamics around Singapore's northern coast, where there is no exchange of seawater across the causeway. S. Buloh mangrove has been used in the past as a site for prawn aquaculture before being declared a protected nature reserve in 1989. S. Khatib Bongsu is not a protected nature conservation site.

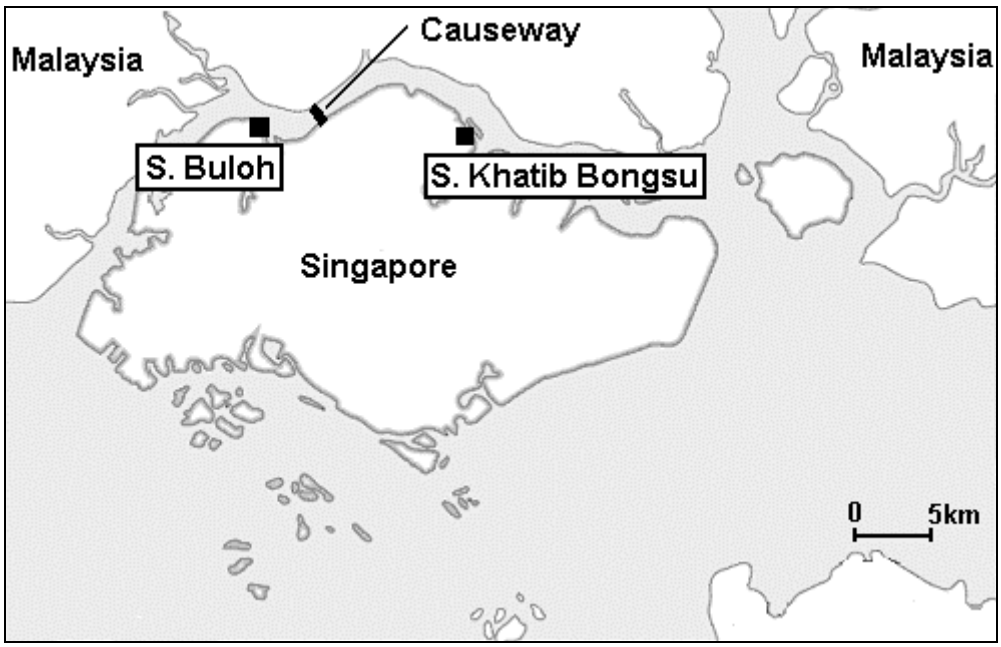

Figure VI-1: Location of Sungei Buloh and Sungei Khatib Bongsu mangroves in Singapore.

VI – 2 – 2 Sample collection and preparation

Species names of organisms collected from the two mangrove sites are presented in Table VI-1. All samples were collected at low tide. Identification and characteristics of their habitat and feeding habits was established in collaboration with the Raffles Museum for Biodiversity (Singapore) and are detailed in Table VI-1. Polychaetes were identified and sampled by Dr Lu Lin of the Tropical Marine Science Institute. Criteria for species selection included large availability and trophic position in the mangrove food web.

Details on the number of organisms collected, their size, and the organs selected for analysis are presented in Appendix C. Fish were caught using a cast net. Other organisms were collected directly from their natural habitat. The number of individuals caught was different for each location and varied between 25 to 50 individuals for polychaetes, 6 to 40 individuals for mollusks, 5 to 170 individuals for crustaceans, and 1 to 20 individuals for fish. All species sampled were available in large numbers, and a total of fifteen species were common to both sites.

Size and age are important parameters affecting the level of POPs accumulation (Stapleton et al., 2001), and organisms were selected in the most similar and available size range possible. After field collection, samples were transported in polyethylene bags packed in ice to the laboratory for processing.

Common name	Scientific name	Habitat	Feeding habit
Green algae	*Chaetomorpha gracilis*	forest floor	primary producer
Red algae	*Catellana sp.*	forest floor	primary producer
Nereid worm	*Neanthes glandicincta*	mudflat	carnivorous
Tube worm	*Diopatra neapolitana*	mudflat	filter-feeder
Nerite snail	*Nerita lineata*	tree trunk/root	algae
Drill shell	*Thai gradata*	tree trunk/root	carnivorous (bivalve/barnacle)
Rodong shell *	*Telescopium telescopium*	streamlet/ prawn pond	detritivore/algae
Green mussel *	*Perna viridis*	rock/pillar	filter-feeder
Lokan (clam) *	*Polymesoda expansa*	buried in mud /prawn ponds	filter-feeder
Leaf oyster	*Isognomon ephippium*	rock, roots	filter-feeder
Mangrove oyster *	*Crassostrea spp.*	rock/pillar	filter-feeder
Barnacles	*Balanus spp*	tree trunk/root	filter-feeder
Snapping prawn *	*Alpheus microrhynchus*	streamlet/tidal pool	carnivorous
Marine prawn *	*Penaus spp.*	estuary	detritivore
Tree climbing crab *	*Episesarma spp*	tree	leaf-eater
Thunder crab *	*Myomenippe hardwicki*	mudflat, under rock or in hole	predator
Half-beak	*Zenarchopterus buffonis*	estuary, pelagic	bivalve/ insects
Giant mudskipper *	*Periophtalmodon schlosseri*	forest, burrow	crab/worm/insect

Table VI-1

(Table IV-1 continued)

Common name	Scientific name	Habitat	Feeding habit
Green spotted goby	*Acentrogobius janthinopterus*	estuary	carnivorous
Glass perchlet	*Ambassis vachellii*	mid-water	omnivorous
Mangrove cardinalfish	*Apogon hyalosoma*	mid-water	omnivorous
Mullet *	*F. mugilidae*	mid-water	omnivorous
Archer fish	*Toxotes jaculatrix*	surface water	carnivorous (fish insects)
Green chromide	*Etroplus suratensis*	mid-water	omnivorous

Table VI-1: Habitat and feeding behaviour of mangrove biota organisms collected in Singapore in April 2004 (adapted from Ng and Sivasothi, 1999). * Species commonly part of human consumption in South-east Asia.

In the laboratory, algae were rinsed with ultrapure water. Polychaetes were depurated in clean seawater for 2 hours and then rinsed with ultrapure water to remove seawater and/or residual sediments. For molluscs, barnacles, prawns and crabs, the whole soft tissues were extracted from the shell for analysis (Boon et al., 2002; Voorspoels et al., 2003). Fish were filleted and meat was collected (both sides of the backbone). When available in an adequate amount, liver (fish) and eggs (fish/crab) were collected for analysis. Each individual chromide and archer fish collected was analyzed separately. All other samples were pooled to form a single batch sample for each mangrove location.

VI – 2 – 3 POPs analysis in biota samples

All samples have been analyzed following the procedure presented in Sections III-3 to III-5, Chapter III, using 25 mL of n-pentane-DCM (1:1 v/v) as the extraction solvent. Lipids were

determined gravimetrically following the procedure presented and validated in Chapter IV. POPs analysis was conducted using GC program 3 (See Section III-6, Chapter III). In the present study, PBDEs refers to the sum of BDE-47, 99,100, 153 and 154; chlordanes to the sum of α-chlordane, γ-chlordane, heptachlor and heptachlor epoxyde; HCHs to the sum of α-, β-, γ- and δ-HCH; endosulfans to the sum of endosulfan I, II and endosulfan sulfate. Details on the congeners analysed for DDTs and PCBs are presented in Section III-6, Chapter III. PCB congeners 55 and 61 were used as recovery standards in all samples. The standard reference material SRM 2978 - green mussel tissue - was analyzed to validate the complete analytical method. Duplicate analysis was performed for each sample.

VI – 2 – 4 POPs in seawater and mangrove sediments

Two and three seawater samples (5 litres each) were analysed for S. Buloh and S. Khatib Bongsu mangroves respectively. Levels of POPs in seawater were measured by Oliver Wurl, using a validated method as described in Wurl and Obbard (2004a). Sediments were collected at three positions in each mangrove simultaneously with biota. Batches of 200 g of sediments were collected between 2 and 7 cm below the surface three times at each position. These three batches were dried in oven at 40°C and mixed in equal proportion in weight to create composite sediments batches (totally three composites per sites). Analytical procedure for POPs quantification in sediments and quality assurance are presented elsewhere (Wurl and Obbard, 2004b).

VI – 3 Results

VI – 3 – 1 Method performance and quality control

Average recovery for recovery standards for POPs was 97% ± 15%. The certified values for duplicate standard reference material SRM 2978 were achieved, with an average recovery of 102% ± 24% for PCBs, DDTs and chlordanes. Typically, there was less than a 30% difference between analytical and certified values, except for trichloro-PCBs (60%). Limits of detection for the method ranged from 0.04 to 0.5 ng/g ww for HCHs, 0.02 to 0.04 ng/g ww for chlodanes, 0.04 to 0.05 ng/g ww for DDTs, 0.05 to 0.4 ng/g ww for endosulfans, to 0.01 to 0.02 ng/g ww for PBDEs, and 0.02 to 0.1 ng/g ww for PCBs according to the congener. Quality assurance data for seawater and sediment analysis are presented in Wurl and Obbard (2004a; 2004b).

VI – 3 – 2 POPs level in seawater and sediments

Levels of POPs in seawater and sediments in the mangroves are summarized in Table VI-2. All POPs, except PBDEs, were detected in subsurface seawater and sediments. PCBs were the dominant POPs in subsurface seawater (0.1 to 6.7 ng/L). Levels of POPs were higher in seawater at S. Buloh, but higher in sediments at S. Khatib Bongsu. The PCB congener profile in seawater was dominated by PCB 151 which represented more than 80% of the total load at both mangroves. HCHs were the dominant OCP in subsurface seawater (0.1 to 2.3 ng/L). *p,p*'-DDT and β-HCH dominated the DDT and HCH profiles of seawater at both sites. The PCB congener profile in mangrove sediments was dominated by tri-chlorinated biphenyls in S. Buloh (66% of the total PCB load), whereas hexa-chlorinated (54%) and penta-chlorinated

biphenyls (27%) were dominant in sediments in S. Khatib Bongsu. HCHs were the dominant organochlorine pesticide in mangrove sediments with levels ranging 1.2 to 6.0 ng/g dw.

	POPs level in sediments (ng/g dw)		**POPs level in seawater (pg/L)**	
	S. Buloh	**S. Khatib Bongsu**	**S. Buloh**	**S. Khatib Bongsu**
PCBs	0.59-1.14 (0.88)	0.80-1.86 (1.33)	6700-7100 (6900)	140-1500 (910)
PBDEs	n.d.	n.d.	n.d.	n.d.
DDTs	<0.1-0.93 (0.29)	0.56-0.85 (0.70)	18-23 (21)	3-72 (32)
HCHs	1.8-6.0 (3.9)	1.2-1.6 (1.4)	2000-2300 (2100)	110-1100 (770)
endosulfan	n.d.	n.d.	280-630 (450)	25-40 (30)
chlordanes	0.01-0.04 (0.02)	0.02-0.06 (0.04)	4-8 (6)	BLD-3 (2)

Table VI-2: Levels of POPs (range and average between brackets) in sediments and seawater from mangroves in Singapore. n.d.: not detected. BLD: below limit of detection.

VI – 3 – 3 POPs level in mangrove organisms

The moisture content of the mangrove biota samples ranged 63-94% and is reported in Appendix C to allow conversion between dry and wet weight units. Lipid content ranged from BLD to 18%, where most values were <5%. In-line with the conclusions of Chapter IV-4-2, data should not be converted to lipid weight for a lipid content of <5% as it may introduce discrepancies.

MDLs in biota ranged from 0.01 to 0.5 ng/g ww of tissue depending on the POP analyte, and are in the range of what is reported elsewhere (Hunter et al., 1995). Surrogate PCB congeners

(i.e., congeners 55 and 61) were satisfactorily recovered, ranging from 75 to 125% (mean recovery was 97%±15%). The certified values for PCBs and organochlorine pesticides in SRM 2978 were achieved, with an average recovery of 102% ± 24%.

The concentrations of PCBs, PBDEs, chlordanes, DDTs, HCHs and endosulfans in the mangrove biota are reported on a wet weight basis (ww) in Appendix D. Concentrations in mangrove organisms ranged from BLD to 45 ng/g ww for chlordanes, BLD to150 ng/g ww for DDTs, 0.6 to190 ng/g ww for PCBs, BLD to 9.9 ng/g ww for PBDEs, BLD to 2.8 ng/g ww for HCHs and BLD to 25 ng/g ww for endosulfans. The lowest concentrations of POPs were found generally in the algae species (*C. gracili* and *Catellana sp.*), the tube worms (*D. neopolitana*), the nerite snail (*N. lineata*), prawn species (*A. microrhynchus* and *Penaus sp.*) and the tree-climbing crab (*Episesarma sp.*). The highest concentrations of POPs were found in the soft tissues of the thunder crab (*M. hardwicki*) and the fishes. Levels of POPs in fish liver and fish eggs were generally one to two orders of magnitude higher than in the muscle tissue. On a lipid weight basis, POPs in fish livers ranged from 41 to 270 ng/g lw for chlordanes, 27 to 860 ng/g lw for DDTs, 65 to 1600 ng/g lw for PCBs, 1.8 to 87 ng/g lw for PBDEs, 1.8 to 17 ng/g lw for HCHs and 2 to 350 ng/g lw for endosulfans.

Concentrations of chlordanes, DDTs, PCBs and PBDEs, on a dry weight basis (dw), are summarized for the fifteen species common to both sites in Figure VI-2. Higher concentrations of PCBs and PBDEs were generally found in biota from S. Khatib Bongsu and chlordanes in biota from S. Buloh. However, the differences between the two sites are generally less than three standard deviations and are not significant.

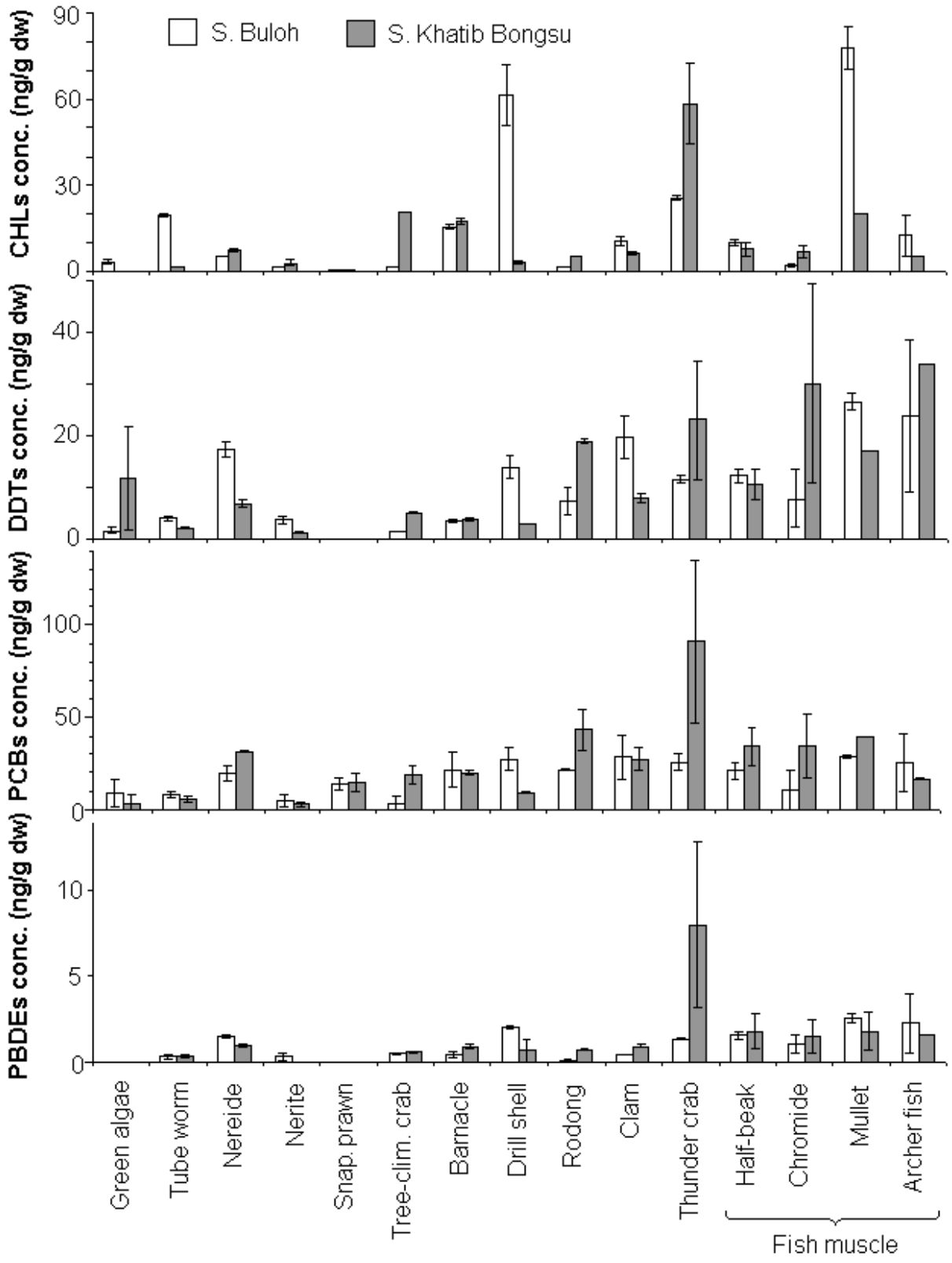

Figure VI-2: Concentrations of POPs in mangrove biota in Singapore (ng/g dw).

VI – 3 – 4 PBDE profile in mangrove organisms

PBDE profiles for mangrove biota samples collected in S. Buloh and S. Khatib Bongsu are presented in figures VI-3 and VI-4 respectively. BDE-47 was the most abundant PBDE congener in the samples accounting for 71 ± 18% of the total PBDE load. The general of contribution to total load is BDE 47 > BDE 99 > BDE 100 > BDE 154 > BDE 153, where BDE 47, 99 and 100 represent 96 ± 11% of the total load. The percentage of BDE-99 as the total PBDE load in polychaetes, rodongs shell, oysters, marine prawns and tree-climbing crabs at S. Khatib Bongsu was significantly higher than all other species at this mangrove site, and all species in S. Buloh (Mann-Whitney, $p<0.05$). Organisms higher in the food chain, especially in S. Khatib Bongsu, appear to have lower levels of BDE-99. BDE-99 was absent in lokan clams (*P. expansa*) at both sites. With the exception of mullet, BDE-47 was present in lower proportions in fish livers than in muscles.

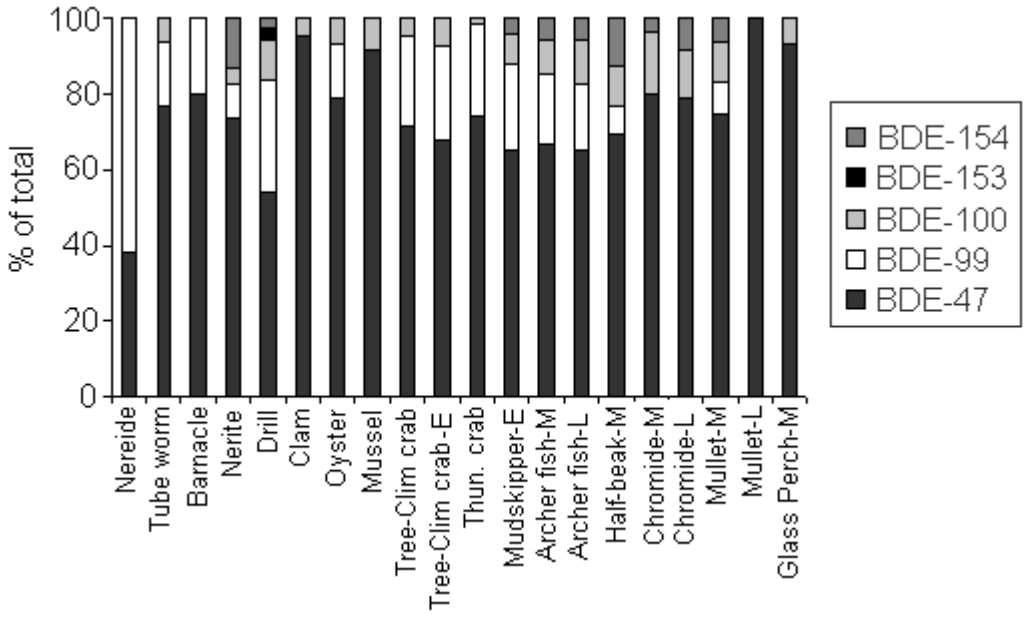

Figure VI-3: PBDE profiles in mangrove biota samples collected in Sungei Buloh. M: muscle. L: liver. E: eggs.

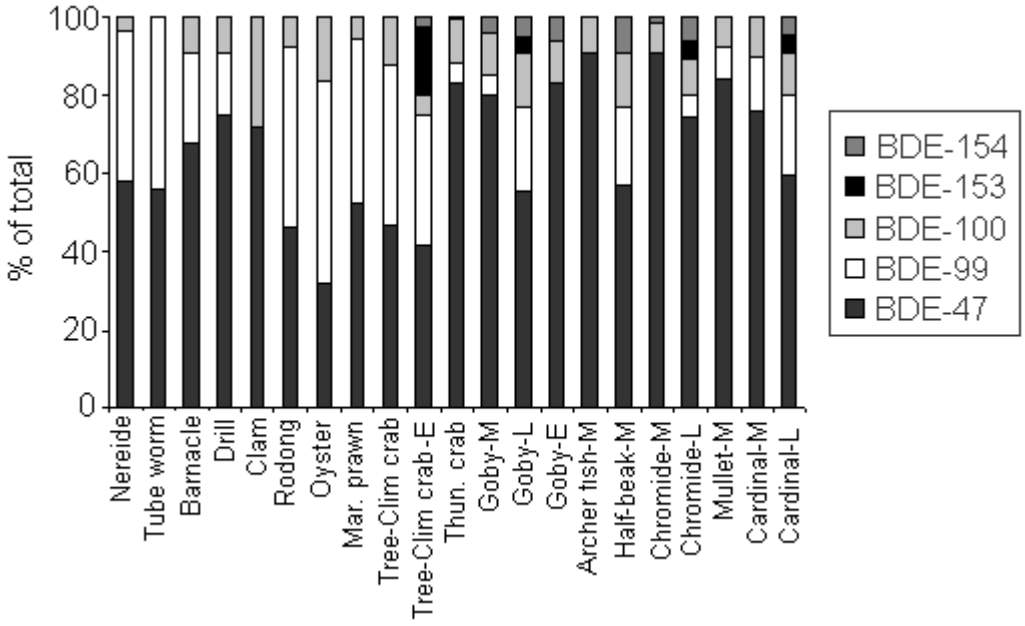

Figure VI-4: PBDE profiles for mangrove biota samples collected in Sungei Khatib Bongsu. M: muscle. L: liver. E: eggs.

VI – 3 – 5 PCB profile in mangrove organisms

Hexachlorinated and pentachlorinated biphenyls dominated the PCB congener profile with an average of 39 ± 12% and 28 ± 10% respectively. PCB profiles were similar at both mangrove sites (See Figures VI-5 and VI-6), where no clear difference in the PCB profile was discernable between the various mangrove species. The closest match in the PCB data for mangrove biota samples collected in this study is Aroclor 1254.

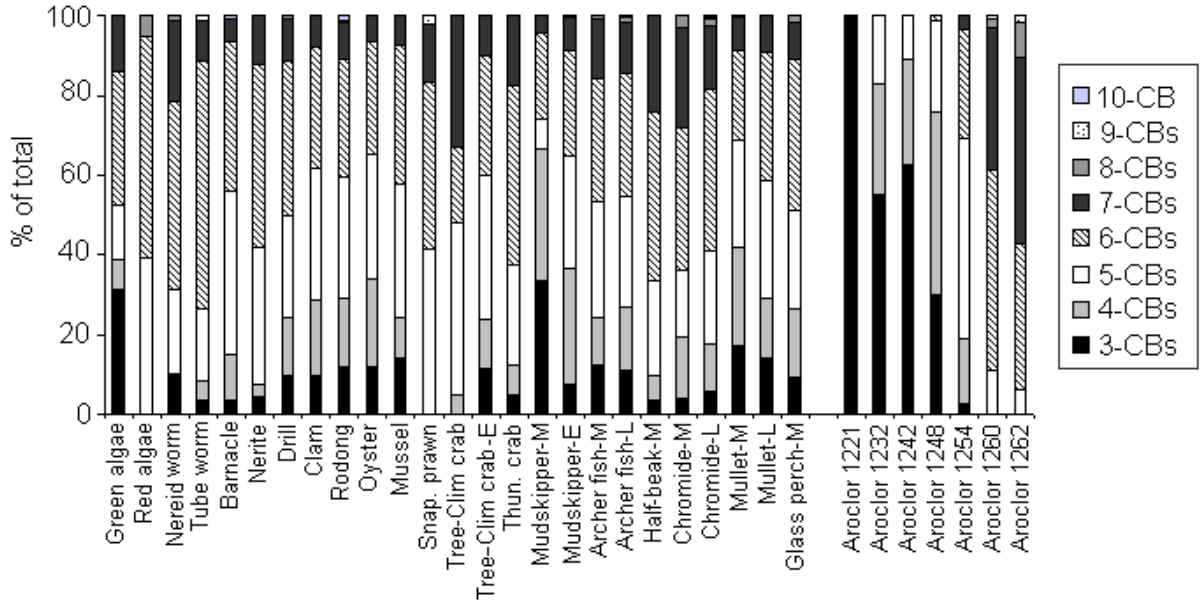

Figure VI-5: PCB profiles in mangrove biota samples collected in Sungei Buloh and in commercial Aroclor products. M: muscle. L: liver. E: eggs.

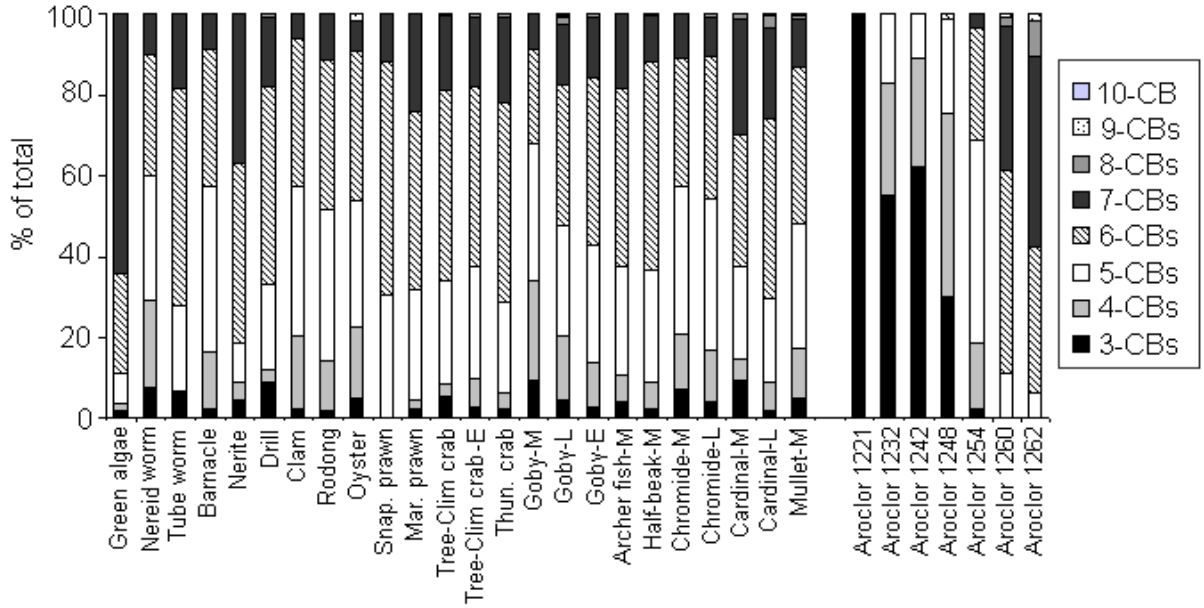

Figure VI-6: PCB profiles in mangrove biota samples collected in Sungei Khatib Bongsu and in commercial Aroclor products. M: muscle. L: liver. E: eggs.

VI – 3 – 6 Chlordane, DDT and HCH profile in mangrove organisms

α- and γ-chlordane generally dominated the chlordane congener profile of mangrove biota with an average of 38 ± 16% and 39 ± 16% respectively (See Figures VI-7 and VI-8). However, a significantly lower percentage of α- and γ-chlordane was found in the two crab species (Mann-Whitney, $p<0.05$), and heptachlor epoxyde dominated the profile in crab species. Gobies, mudskipper and shrimp tissues also contained a significantly higher percentage of heptachlor epoxyde (Mann-Whitney, $p<0.05$).

p,p'-DDE was the dominant DDT congener in all samples, except in the green algae in S. Khatib Bongsu, representing an average of 84 ± 15% of the total DDT load. β-HCH was the dominant HCH congener, representing an average of 72 ± 37% of the total HCH load.

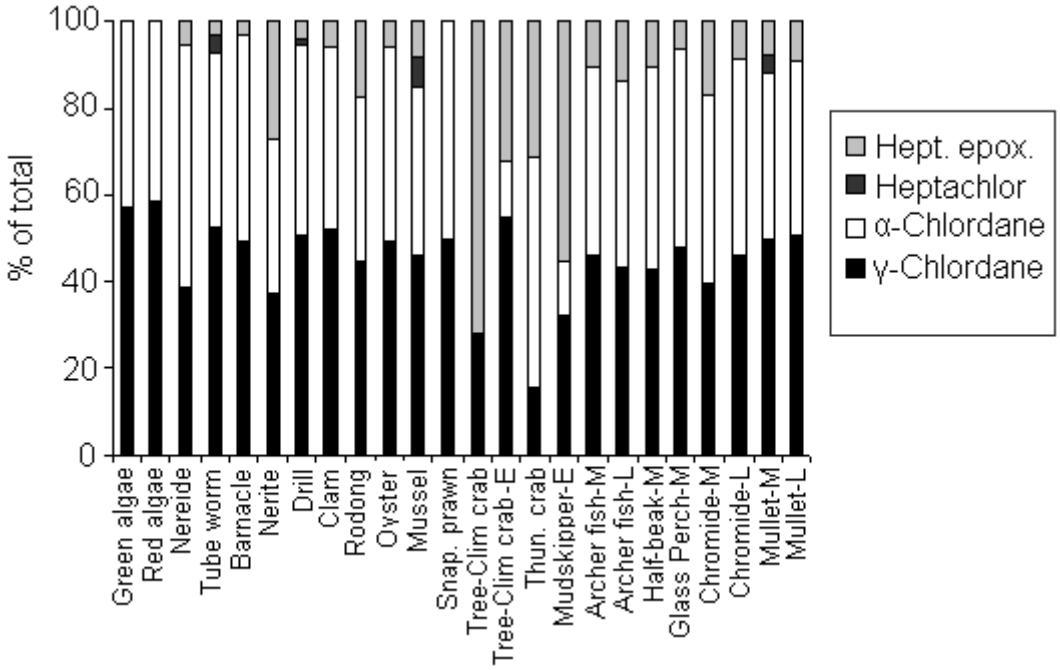

Figure VI-7: Chlordane profiles in mangrove biota samples collected in Sungei Buloh. M: muscle. L: liver. E: eggs.

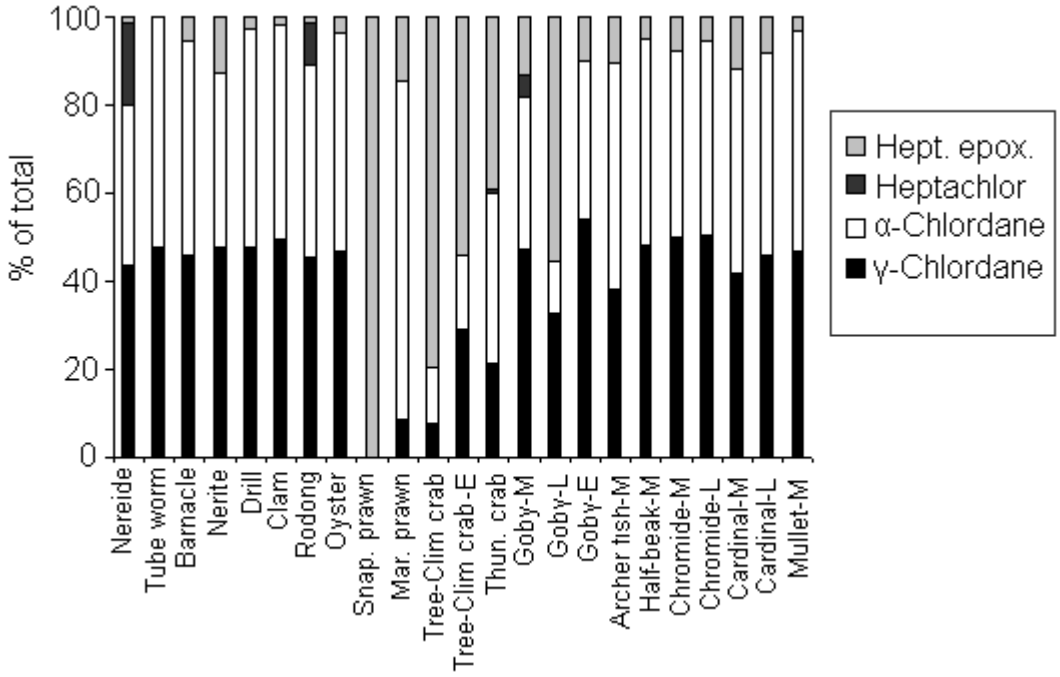

Figure VI-8: Chlordane profiles in mangrove biota samples collected in Sungei Khatib Bongsu. M: muscle. L: liver. E: eggs.

VI – 4 Discussion

VI – 4 – 1 POPs levels and profiles

Overall, the levels of POPs in mangrove sediments can be regarded as low (< ng/g for all analytes), i.e. lower than what has been recorded in the marine sediments in Singapore (Wurl and Obbard, 2004b). Other studies have also reported that mangrove sediments have PCB levels lower or comparable to those in marine sediments, for example in Hong Kong (Tam and Yao, 2002; Zheng et al., 2000). The congener profiles of PCBs and DDTs were different for seawater, sediments and biota samples. HCHs concentrations in seawater samples were relatively high compared to other POPs, but were relatively low levels in biota. The low bioaccumulation of HCHs is expected since their K_{ow} value is below 5 (3.8).

Concentrations of POPs in in food webs of, including PCBs, PBDEs, DDTs and chlordanes, is related to the feeding habits and trophic level of organisms (Dietz et al., 2000; Kidd et al., 2001). The highest POPs concentrations were found in higher trophic predator species (e.g. thunder crabs, drill shell, archer fish, chromide) and detritivores (e.g. rodong shell), while organisms lower at lower trophic levels (tube worm, nerite, prawns) had generally the lowest levels of POPs. In particular, the predator thunder crab (*M. hardwicki*) had higher tissue levels of POPs than leaf-eater crabs (*Episesarma spp*), although organisms were collected from the same habitat. A similar observation is valid when comparing the predator polychaete, *N. glandicincta*, and the filter-feeder tube-worm (*D. Neapolitana*). Differences in POP concentrations between the invertebrates and fish were generally one to two orders of magnitude, which is consistent with studies in other ecosystems (Boon et al., 2002; Dietz et al., 2000). Major biomagnification stage is usually associated between the trophic level of fish and mammals.

The dominant group of POPs in Singapore's mangroves was the PCBs, followed by organochlorine pesticides DDTs and chlordanes. PBDEs and HCHs were present at concentrations generally one or two orders of magnitude less than other PCBs. This observation is consistent with the findings presented in Chapter V using *P. viridis* as a bioindicator of marine contamination.

Comparison of PBDE levels with other studies is more straightforward by limiting the comparison to the single congener always present in the highest concentrations, i.e. BDE-47 (Boon et al., 2002; Voorspoels et al., 2003). The levels of BDE-47 detected in this study

(BLD-5.9 ng/g ww for all mangrove samples; 1.8-69 ng/g lw in fish livers) cover the middle range of data reported from Europe, Japan and Canada (De Wit, 2002; Law et al., 2003; Voorspoels et al., 2003). Levels of BDE-47 in mangrove fish muscles were higher, or in the range, of levels recorded for tuna muscle tissue in the China Sea (Ueno et al., 2004). The prevalence of BDE-47 in the PBDE profile, and the relative percentages of other PBDE congeners, are consistent with previous studies in other ecosystems (Boon et al., 2002; Voorspoels et al., 2003), suggesting that BDE-47 dominance is independent of the ecosystem studied. BDE-47 was present in lower proportions in the liver of mangrove fish compared to muscle tissue, which is consistent with a previous reports on marine fish in Belgium (Voorspoels et al., 2003), but no clear explanation is available. Higher proportions of BDE-99 were observed in the PBDE profile of organisms at lower trophic levels in S. Khatib Bongsu (i.e. polychaetes, oyster, rodong shell, marine prawn, tree-climbing crab). In Chapter V, BDE-99 levels in green mussel tissues were reported as greater than those for BDE-47 in samples collected in Punggol, i.e. near S. Khatib Bongsu, but not mussels collected in S. Buloh. The source of PBDE contamination observed in Punggol is likely to also impact the mangrove in S. Khatib Bongsu. As a result, organisms at low trophic levels in S. Khatib Bongsu (polychaetes, oysters, rodong shell, marine prawn and tree-climbing crab), with a high uptake and/or poor metabolism capability of PBDEs, will also have higher levels of BDE-99. Organisms at higher trophic levels (e.g. thunder crab, fish) may have lower loads of BDE-99 as a result of different uptake/metabolic processes. Gustafsson et al. (1999) showed that the uptake of BDE-47 and 99 were virtually identical for the mussel *M. edulis*. However, Stapleton et al. (2004) demonstrated, in a controlled experiment, a rapid assimilation of BDE-47 by juvenile carps, whereas no assimilation of BDE-99 was observed. Such

differences may help to explain observed PBDE patterns in the mangrove organisms of S. Khatib Bongsu.

PCBs concentrations found in shrimps and fish in the mangroves of Singapore were similar to those reported in Hong Kong mangroves (Liang et al., 1999). PCB concentrations in mangrove fish correspond to the upper range of what has been recorded in various species of fish elsewhere in the Asia-Pacific region (UNEP, 2002b). The PCB profile was generally dominated by penta and hexachlorinated biphenyls, matching the profile of the commercial product Aroclor 1254. The same PCB profile was observed in the soft tissues of green mussels collected in Singapore, reinforcing the hypothesis of Askarel use being the source of PCB contamination in Singapore (See Section V-4-2, Chapter V).

Concentrations of chlordanes (sum of α- and γ-) were higher than reported values for other marine food webs in the Baltic Sea (Falandysz et al., 2001). Chlordane concentrations in mangrove fish correspond to the upper range of what has been recorded in various species of fish elsewhere in the Asia-Pacific region (UNEP, 2002b). The chlordane profile was dominated by α- and γ-chlordane in all samples except for crabs and gobies. Falandysz et al. (2001) also noted differences in chlordane levels between crabs and other marine organisms which were attributed to the potency of crabs to metabolize chlordane into oxychlordane.

DDT concentrations in mangrove fish correspond to the middle range of what has been recorded in various species of fish elsewhere in the Asia-Pacific region (UNEP, 2002b). *p,p*'-

DDE was the dominant DDT congener, suggesting that no input of DDT occurred to these ecosystems recently.

VI – 4 – 2 Site comparison

Many biological and environmental parameters affect the uptake of contaminants in marine biota. Therefore, a simple interpretation of pollutant data for a large number of organisms is not readily possible when comparing the two mangrove sites studied. For example, organisms at S. Khatib Bongsu were generally smaller in size than those at S. Buloh (See Appendix C). As POP concentrations are known to increase with size in some species, such as fish (Liang et al., 1999; Makareviscz et al., 2003), the comparison of the two sites may include a size-related bias. However, mangrove organisms collected in S. Khatib Bongsu generally have higher levels of PCBs and PBDE. This observation is consistent with the findings of Chapter V where *P. viridis* was used as a bioindicator, as higher concentrations of PCBs and PBDEs were recorded in the stations in the east Strait of Johore compared to the west.

VI – 4 – 3 Risk for higher trophic levels

Levels of POPs in marine mammals and birds liver are generally one to three orders of magnitudes higher than in fish liver which, in turn, are four orders of magnitude lower than human adipose tissues (Dietz et al., 2000; Liang et al., 1999). Mammals, such as smooth-coated otters (*Lutrogale perspicillata*), and over 100 species of birds have been recorded in the mangroves in Singapore (Ng and Sivasothi, 1999). If we use this species richness as a guide for mangrove food webs in Singapore, POPs levels in the μg/g ww range are predicted

to be present in bird and mammal livers. The analysis of POPs in the eggs of fish-eating birds in Hong Kong, such as heron and egrets, revealed toxicological risks to breeding success (Connell et al., 2003). As levels in the present studies are comparable to those in Hong Kong, it is recommended that the risk for organisms at higher trophic levels in the mangroves of Singapore is evaluated.

As noted in Table VI-1, at least ten species of organisms analysed in the present study are commonly consumed by humans in South-east Asia. Concentrations of POPs did not exceed the food safety standards of Singapore (See Table II-4, Chapter II). However, PCB concentrations in mangrove fish muscles are also higher than the mean levels of PCBs in seafood commonly consumed in Singapore, which was found to increase the cancer incidence risk over a human lifetime (See Section IX-2, Chapter IX). Therefore, consumption of organisms from mangroves of Singapore should be considered carefully.

VI – 5 Conclusion

The present study confirms the ubiquity of POPs, including PBDEs, in the marine environment of Singapore. A biomagnification phenomenon was observed amongst the species collected and analysed from both mangrove sites studied. Further assessment is recommended on organisms at higher trophic levels in the mangrove food web, including mammals and birds due to the potential for ecotoxicological impacts. The ubiquity of these pollutants in Singapore's marine environment supports the need for a greater awareness of bioaccumulation processes, particularly for organisms cultured locally and destined to human consumption.

CHAPTER VII – EXPOSURE OF AQUACULTURE OYSTERS TO POLLUTANTS IN SINGAPORE'S COASTAL WATER

VII – 1 Introduction

Marine pollution represents a potential risk for the aquaculture industry as seafood exposed to contamination, even at trace levels, may not be fit for human consumption. Additionally, pollution can present a direct threat to aquaculture business as by impairing production yields and the health of aquacultured organisms (Mc Dowell Capuzzo, 1996; Sugawara and Okoshi, 1993).

The Pacific oyster, *Crassostrea gigas*, is cultured in the Asia-Pacific Region, but also in Europe, America and Africa. World production reached 4.1 millions of tons in 2001, with 85% of the total produced in China (FAO, 2001). As a filter feeder, *Crassostrea gigas* has been used as a bioindicator for anthropogenic contaminants (Gunther et al., 1999; Hunter et al., 1995; Mc Dowell Capuzzo, 1996). The Pacific oyster accumulates POPs to levels similar, or greater, than mussels. However, high mortality rates have been observed in monitoring studies using transplanted organisms, which can undermine the value of the data collected (Gunther et al., 1999). A bioassay using Pacific oyster embryo larvae has been developed to monitor water discharges in the marine environment in the UK (De Fur et al., 1999).

The present study examined the comparative growth rates of Pacific oysters at two sites in Singapore, one 'clean' and one 'contaminated'. POPs bioaccumulation in *C. gigas* was

monitored continuously from the juvenile stage until organisms reach their market size to assess potential health risks of shellfish consumption. The study also investigated the transplantation of 'mature' organisms between the 'clean' and the 'contaminated' sites and vice-versa to determine oyster response to contamination and ability to recover from pollutant exposure.

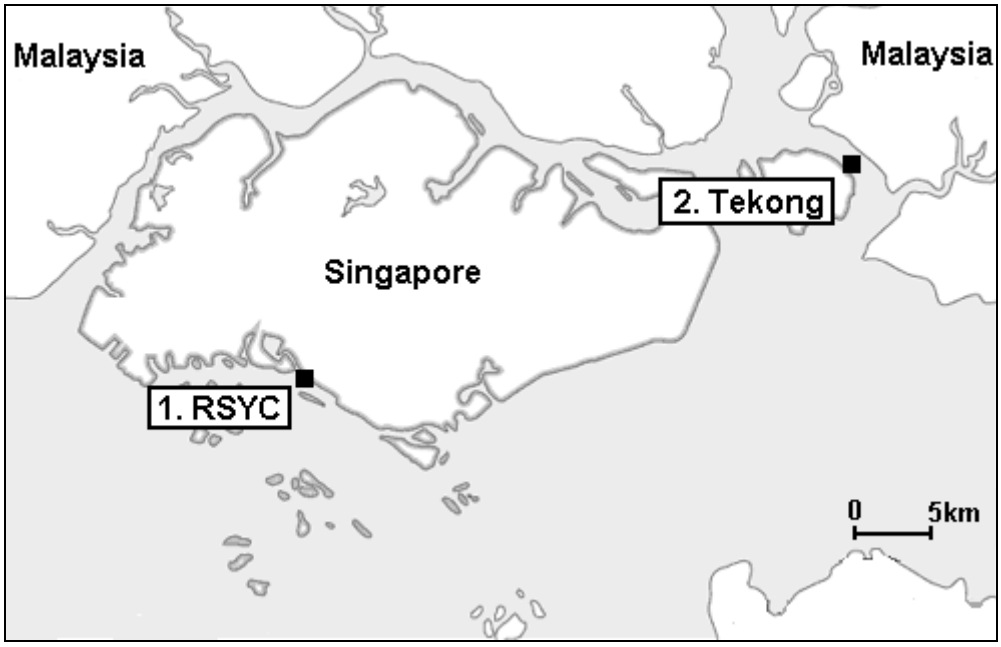

Figure VII-1: Locations of the two sites in Singapore's coastal environment where *Crassostrea gigas* were grown.

VII – 2 Materials and Methods

VII – 2 – 1 Experimental design

In this study, the growth of oysters was compared at two sites of Singapore's marine environment between July 2003 and May 2004 (See Figure VII-1). The first site, the Republic of Singapore Yacht Club (RSYC) marina, is located near the shipping area in West

Coast (N 01°17.60' E 103°45.00'). The second site is a fish farm near the island of Pulau Tekong (N01° 25.82' - E104° 03.97'). The sites were chosen for their marked difference in prevailing levels of POP contamination, as described in Section V-3-3, Chapter V. Seeds of *Crassostrea gigas* were imported from Australia. In a first attempt (July 2003), oysters were obtained of an initial size averaging 5±1 mm. However, the survival rate was not satisfactory (<15%) at both sites. In September 2003, a second batch of oyster seeds was purchased at an average size of 6±1 mm and was acclimatized for 3 weeks in the fish farm in P. Tekong (See Figure VII-2). After 3 weeks, the survival rate was >95%, and 1,000 oysters were then transferred to RSYC (Day 0); whilst another 1,000 oysters remained in P. Tekong.

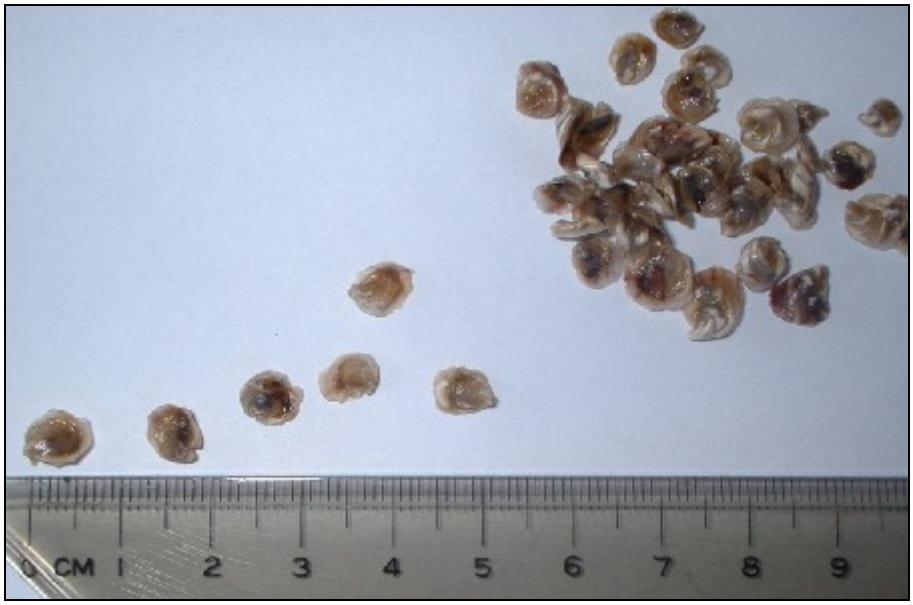

Figure VII-2: Seeds of *Crassostrea gigas* at the initial stage of the experiment.

Oysters were grown in cages at constant depth (between 0.5 and 1 m) for the duration of the experiment i.e. 230 days. Maintenance included monthly cleaning of the cage (removal of biofouling organisms) and size-grading of the oysters. At the initial stage of their growth

(size < 20 mm), oysters seeds were grown in mesh nylon bags of mesh size 2 mm, containing up to 1,000 oysters. As the size increased, oysters were transferred to bags of a larger mesh size (8 mm) and finally to the cage itself (mesh size 15 mm). The number of oysters per cage was reduced over the remaining duration of the experiment to avoid overcrowding and competition between organisms. The number of surviving oysters, and their size, at each site was recorded monthly.

As explained in greater detail below, strong differences were observed between the two sites in terms of oyster growth and contaminant load. As a result, in February 2004, i.e. 126 days after the beginning of the experiment, half of the remaining oysters from each site were transferred to the other site, in order to study the organism's response to a changed environment.

VI – 2 – 2 Water quality

Physical parameters, including temperature, salinity, dissolved oxygen and total organic carbon were monitored monthly at both sites by the Marine Port Authority (Private communication). Levels of POPs in seawater were monitored simultaneously at the same sites by co-workers (Wurl and Obbard, 2004a).

VII – 2 – 3 Sample collection and chemical analysis

Each month between October 2003 (Day 0) and May 2004 (Day 230), 100 oysters were collected from each site for analysis. Samples were separated into distinct class sizes for analysis (i.e. <25 mm, >25-45 mm, >45-65 mm and >65 mm). Samples were transported to

the laboratory in polyethylene bags in ice-boxes for analysis. To prepare samples for POP analysis, soft tissues were removed from the shell and homogenized in a stainless steel blender. The size, total weight and weight of the soft tissues were recorded for each organism. Homogenized samples were then frozen at –20 °C.

All oyster tissue samples were analyzed following the procedure presented in Section III-3, Chapter III, using 25 mL of n-pentane-DCM (1:1 v/v) as the extraction solvent. POPs analysis was conducted using GC program 3 (See Section III-6, Chapter III). Quality control included spiking of surrogate PCB 55 and 61 standards to samples, analysis of standard reference material SRM 2978 and procedural blank analysis. Two to eight oyster samples were analysed for the P. Tekong site at each sampling date. Due to the small amount of tissue present in samples taken from RSYC, only one or two replicates could be analysed at each sampling date. In the present study, PBDE concentrations reported refer to the sum of BDE-47, 99 and 100. Details on the congeners analysed for chlordanes, DDTs and PCBs were presented in Section III-6, Chapter III. Four oyster samples were sent to an external laboratory for tributyl-tin analysis. These samples correspond to the largest size range of oysters collected at both site on Day 126, and transferred oysters on Day 168 at both sites.

VII – 3 Results

VII – 3 – 1 Water quality

Physical parameters and POPs levels in subsurface seawater between October 2003 and May 2004 are presented in Table VII-1.

Parameter	RSYC	P. Tekong
Temperature (°C) [a]	30 ±1	29±1
Salinity ($^0/_{00}$) [a]	32±2	29±3
Dissolved oxygen (mg/L) [a]	6.0±1.5	4.8±1.0
Total organic carbon (mg/L) [a]	9.3±9.3	13±11
PCBs (pg/L)[b]	940±780	290±190
DDTs (pg/L)[b]	160±130	23±10
chlordanes (pg/L)[b]	65±59	<20
heptachlor (pg/L)[b]	190±270	<16
PBDEs (pg/L)[b]	<80	<80

Table VII-1: Comparison of physical and chemical properties of seawater at the two sites of oyster culture. [a] Average for surface water collected between September 2003 and May 2004. [b] Average for data collected between October 2003 and May 2004.

Overall, there were no significant difference in term of temperature, salinity, dissolved oxygen, total organic carbon between the two sites (Mann-Whitney, $p<0.05$). The levels of

all POPs in subsurface seawater, except for PBDEs, were higher at RSYC compared to P. Tekong. PBDEs were not detected at either site. The typical congener profile for PCBs and DDTs in subsurface seawater is presented in Figure VII-3 and VII-4. Trichlorobiphenyls typically represented 75% of the total PCB load in seawater from RSYC, whereas tetrachlorobiphenyls were prevalent in P. Tekong, accounting for 94% of the total load. For the DDTs, *p,p'*-DDD accounted for a slightly higher proportion of the total DDT present that *p,p'*-DDT and *p,p'*-DDE at both sites, although the difference is not significant.

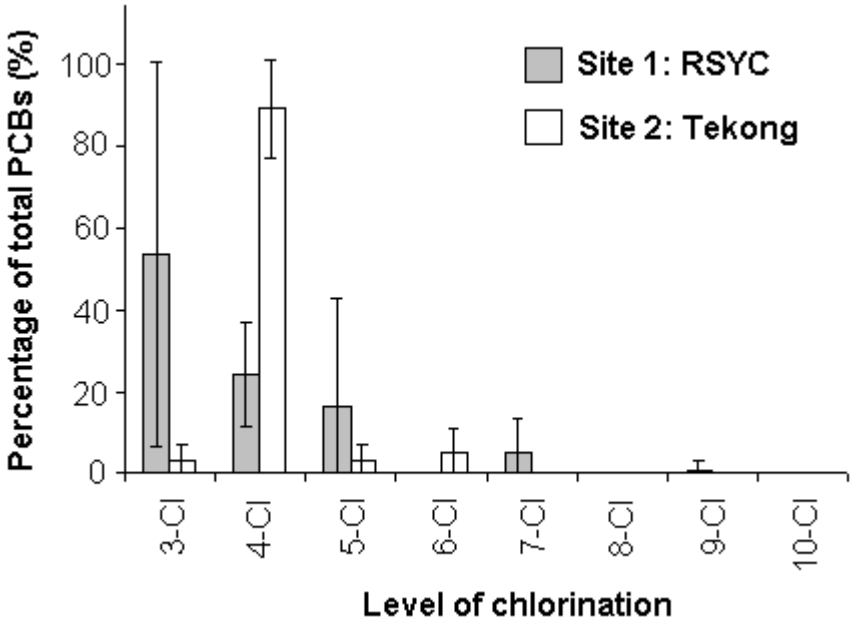

Figure VII-3: Typical PCB congener profile in subsurface seawater (adapted from Wurl and Obbard, 2004a).

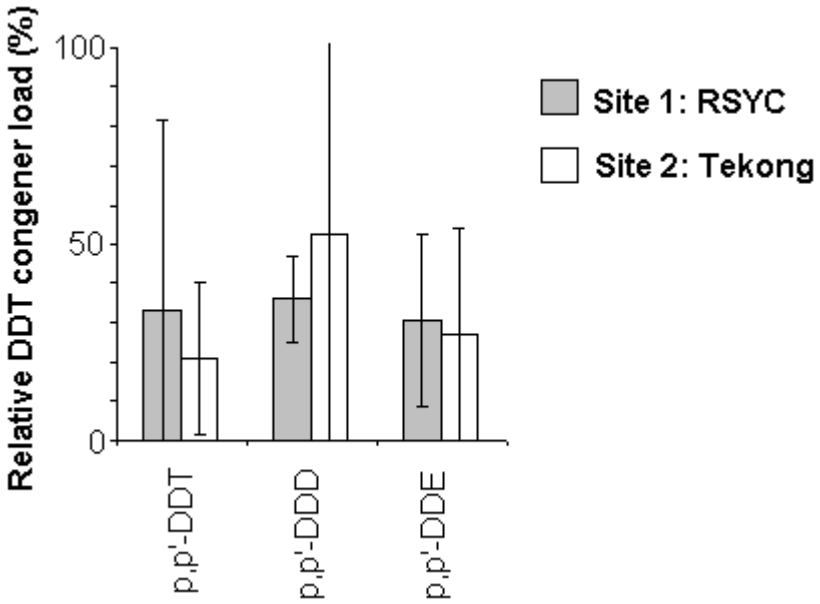

Figure VII-4: Typical DDT congener profile in subsurface seawater (adapted from Wurl and Obbard, 2004a).

VII – 3 – 2 Growth and morphological characteristics

Oyster survival rates were similar for both P. Tekong (81%) and RSYC (83%) over the 230-day experiment. However, growth rates were different between the two sites (See Figure VII-5). The oysters in P. Tekong grew from 11 to 68 mm over 230 days - most of them reaching their market size (i.e. >70 mm) at the end of the experiment. On the contrary, the oysters at RSYC grew relatively slowly, from 11 to 20 mm over 230 days. On day 126, oysters from each site were exchanged. Figure VII-5 shows that the oysters initially present at RSYC and transferred to P. Tekong started to grow more vigorously, and eventually reached 38±12 mm on day 230. In contrast, the growth of the oysters initially at P. Tekong and transferred to RSYC slowed considerably compared to the oysters remaining at P. Tekong.

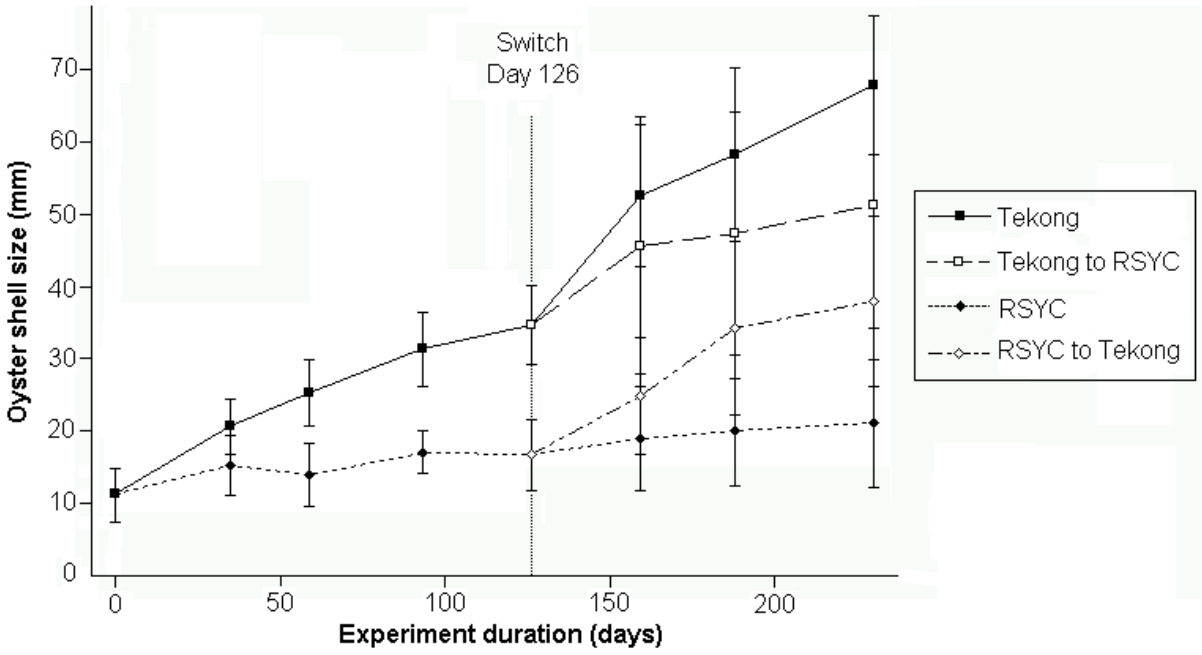

Figure VII-5: Shell size (mm) of cultured oysters at RSYC and Pulau Tekong over 230 days. On day 126, some oysters were swapped locations.

In addition to radical differences in shell growth rates between the two sites, morphological changes were also observed in oysters grown at RSYC. From the start of the experiment, *C. giga*s oysters cultured at RSYC developed an abnormal shell morphology, in which the valve thickened and eventually become ball-shaped (See Figure VII-6a bottom, b, c). On the contrary, oysters cultured in P. Tekong developed a normal shape (Figure VII-6a top). After the exchange of location, on day 126 of the experiment, oysters initially at RSYC and transferred to P. Tekong started to grow normally (See Figure VII-5d). On the contrary, mature oysters transferred from P. Tekong to RSYC displayed the same shell-thickening symptoms as the oysters initially cultured at RSYC (Figure VII-6b and c).

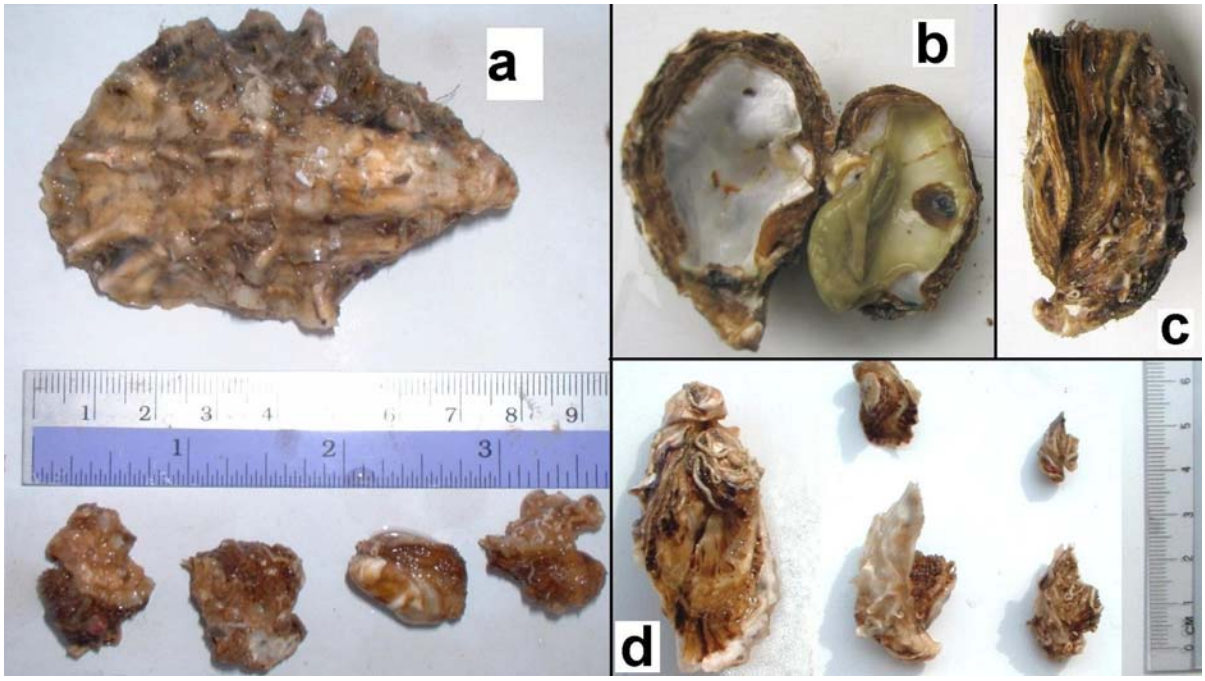

Figure VII-6: Images of (a) oysters at uncontaminated (top) and contaminated (bottom) after 230 days, (b) & (c) show mature oysters transferred from uncontaminated to contaminated site after 100 days and (d) oysters transferred from contaminated to uncontaminated site after 100 days. Chambering effect was observed in oysters from the contaminated site (Figures a bottom; b & c). After transplantation from the contaminated to the uncontaminated site, oysters started to grow normally again, without sign of chambering effect anymore (d).

The tissue yield, i.e. the weight of soft tissue divided by the total weight expressed as a percentage, was calculated for the various batches of oysters. The tissue yield was higher in oysters grown at P. Tekong compared to RSYC with an average yield of 20±4% versus 11±4% at RSYC (See Figure VII-7). The yield at RSYC decreased steadily over the 230-day period to eventually reach only 7% of the initial value on day 230. After the switch of location, the oysters initially in RSYC and transferred to P. Tekong recovered and reached the same tissue yield as organisms initially in P. Tekong after another 100 days. On the contrary, the yield of mature oysters transferred from P. Tekong to RSYC decreased and eventually reached the same yield as organisms initially cultured at RSYC after 100 days.

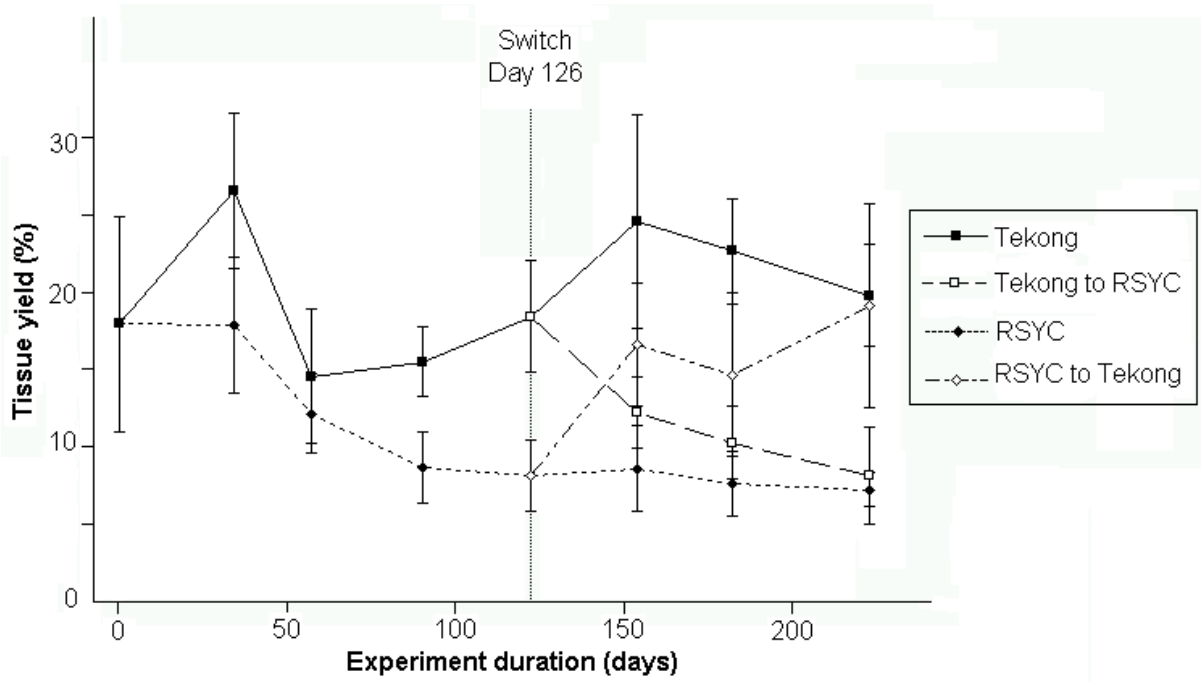

Figure VII-7: Tissue yield (%) of cultured oysters at RSYC and Pulau Tekong over 230 days. On day 126, some oysters were swapped locations.

VII – 3 – 3 POPs burden in oysters

MDLs ranged from 0.04 to 0.3 ng/g ww depending on the POP analyte, and are in the range of what is reported elsewhere (Hunter et al., 1995). Surrogate PCB congeners (i.e., congeners 55 and 61) were satisfactorily recovered, ranging from 75 to 125% (mean recovery was 87%±14%). The certified values for PCBs and organochlorine pesticides in SRM 2978 were achieved, with an average recovery of 89% ± 19%. Levels of POPs in oysters cultivated at RSYC and P. Tekong are presented in Figure VII-8.

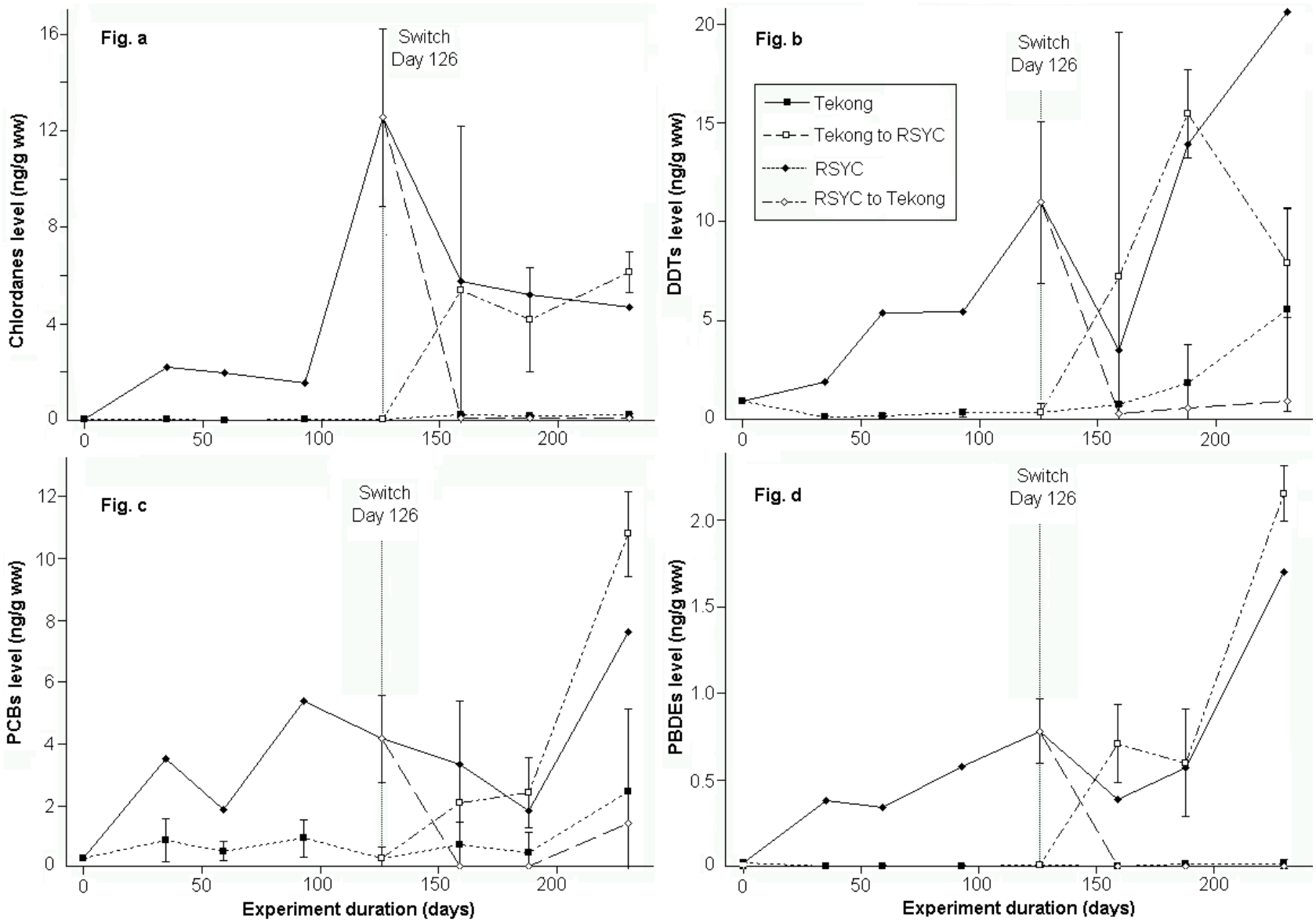

Figure VII-8: Levels of chlordanes (a), DDTs (b), PCBs (c) and PBDEs (d) in *C. gigas* cultured in Singapore (ng/g ww).

Levels of POPs in oyster tissues ranged from <0.04 to 17 ng/g ww for chlordanes, from <0.1 to 32 ng/g ww for DDTs, from <1.2 to 11.7 ng/g ww for PCBs and from <0.07 to 2.2 ng/g ww for PBDEs. Levels of POPs were higher in oyster tissue from RSYC than in P. Tekong, with a factor ranging 22 to 250 for chlordanes, 5 to 30 for DDTs, 3 to 17 for PCBs and 55 to 200 for PBDEs over the 230 day experiment, excluding the exchange period. After the switch of location, the oysters initially at P. Tekong and transferred to RSYC accumulated POPs levels similar to oysters initially cultured in RSYC after only 33 days. On the contrary, levels of POPs in oysters transferred from RSYC to P. Tekong decreased over time, and reached the levels found in organisms initially cultured at P. Tekong within 33 days.

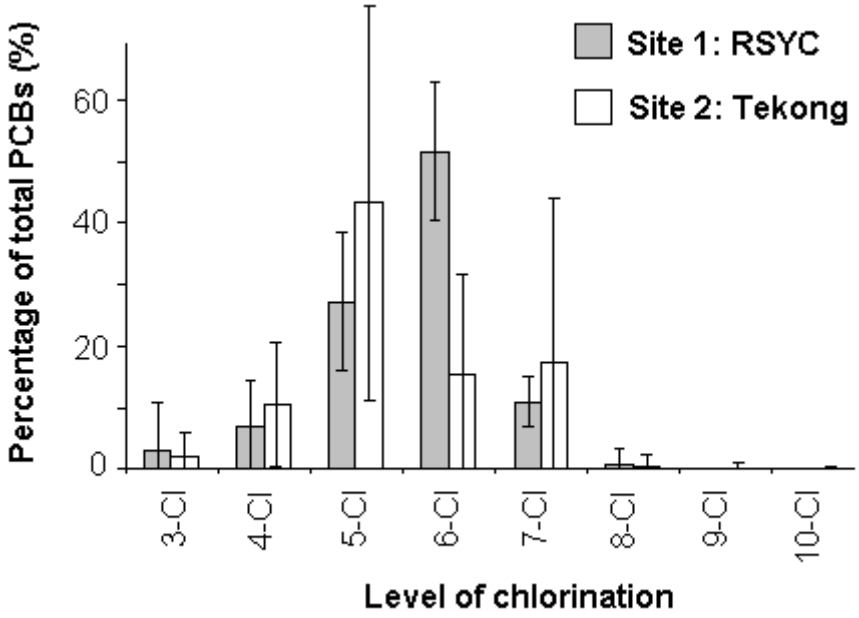

Figure VII-9: Typical PCB congener profile in oyster's tissues at both sites.

A typical PCB and DDT congener profile in the oyster tissues is presented in Figure VII-9 and VII-10. Penta- and hexa-chloro biphenyls dominated the PCB profile in oyster tissues, and typically represented between 27 and 51% of the total PCB load from RSYC, and

between 15 and 43% at P. Tekong. For PBDEs, BDE-47 was the dominant congener found in the oyster tissues at both sites. As for DDTs, all three congeners were present in almost identical amounts in oysters at RSYC (See Figure VII-10). In P. Tekong, *p,p*'-DDE was dominant, although differences are not significant.

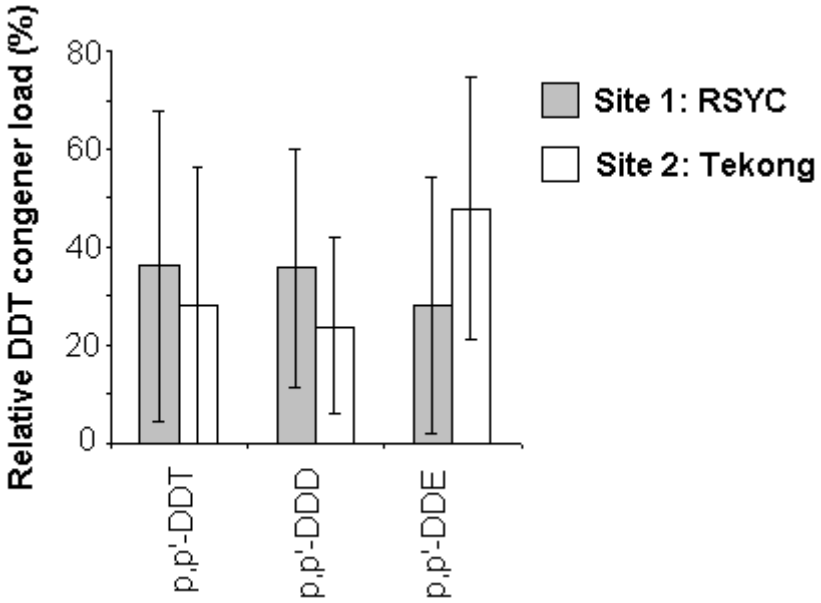

Figure VII-10: Typical DDT congener profile in oyster's tissues at both sites.

VII – 3 – 4 Analysis of TBT in oysters

TBT levels reached 130 ± 20 ng/g ww in mature oysters from P. Tekong and 115 ± 30 ng/g ww in oysters transferred from this site to the RSYC. The difference in TBT content of oyster tissues was therefore not significant, despite strong morphological changes in the organisms from the contaminated site.

VII – 4 Discussion

VII – 4 – 1 POPs burden

The higher POP levels recorded in oyster tissues at RSYC relative to those in the P. Tekong corroborate the findings of a previous study using the green lipped mussel, *P. viridis* as a bioindicator (See Chapter V). Levels of POPs recorded in oysters cultured at P. Tekong were low in the context of levels reported in the available literature for elsewhere (Hunter et al., 1995; Oliver et al, 2001). However, levels recorded in *C. gigas* cultured at RSYC for chlordanes (1.5-17 ng/g ww) and DDT (1.9-32 ng/g ww) were in the upper range of values reported, and PCBs (1.8 to 11.7 ng/g ww) were in the middle range. No data are available on the occurrence of PBDEs in oysters, but the concentrations in *C. gigas* reflect the geographical distribution of the flame retardants in *P. viridis* in Singapore's coastal waters in 2002 (See Chapter V). This study confirms that POPs, including PBDEs, are present and bioaccumulate in the biota within the marine environment of Singapore.

POPs levels found in oysters at both sites are below available food safety standards for PCB, DDT and chlordane levels set by the Government of Singapore (1990). There are no available local standards for PBDEs in seafood. Han et al. (2000) reported DDT levels ranging from 1.2 to 68 ng/g ww in oysters cultured in Taiwan's coastal waters, and deduced that high volume consumers of contaminated oysters were exposed to a cancer risk greater than 10^{-6} over a lifetime. In the present study, DDT levels reached 32 ng/g ww in oyster tissues when cultivated at RSYC. This value is in the same range as that reported from

Taiwan, and it is therefore advisable to limit consumption of oysters and other organisms cultured within certain parts of the west coast of Singapore.

PCB congener profiles in the oysters differed from the profile determined in the seawater at both locations. A difference in the PCB profile between oysters and its environment is both common and expected (Mc Dowell Capuzzo, 1996), as the profile can be expected to vary along food chains due to differences in the bioaccumulation and metabolisation of PCB congeners between species (Wong et al., 2004). At the uncontaminated site, DDT metabolites were dominant in both seawater and oysters. As the commercial DDT mixture is principally composed of *p,p'*-DDT, it can be assumed that no recent input of the commercial pesticide occurred near the uncontaminated site. In contrast, *p,p'*-DDT was present in a similar proportion to its metabolites in both seawater and oyster tissues from the contaminated site, suggesting a current bioavailability of the parent DDT compound.

VII – 4 – 2 Oyster's growth

Growth rates of *C. gigas* from P. Tekong are comparable to what has been recorded in aquaculture sites in other tropical countries, such as Brazil (Poli and da Silva, 2004). On the contrary, the growth of the oysters in RSYC was severely impaired. Oyster growth can be affected by numerous parameters, including salinity and temperature (Sugawara and Okoshi, 1993), as well as exposure to contaminants. POPs levels have been correlated with various developmental impairments during larval stages (Konar and Stephenson, 1995), but they may also affect the growth of juvenile and mature oysters through various mechanisms. For example, PCBs were shown to adversely affect the metabolism of *Crassostrea spp.* oysters,

via the alteration of lipid content (Ferreira and Vale, 1998) and glycogen metabolism (Encomio and Chu, 2000). Oliver et al. (2001) established that hemacyte activities in oysters were correlated with the levels of PCBs and some pesticides including DDT, suggesting that POPs may lower the immunological defence of oysters and increase susceptibility to infectious diseases. *C. gigas* metabolism is known to be affected by the presence of other anthropogenic contaminants such as 4-nonylphenol, heavy metals and TBT (Nice et al., 2000; Chen, 1994; Waldock and Thain, 1983). In the present study, physical parameters such as temperature, salinity, dissolved oxygen and total organic carbon were essentially similar at both sites and were probably not related to the oyster growth rate. POPs levels were found to be greater in seawater and oyster tissues from the contaminated site, and are a possible cause of the slower growth rates.

Shell anomalies for bivalves can be attributed to the presence of environmental contaminants, but also to physical parameters in seawater such as particulate loading (Waldock and Thain, 1983). However, the valve thickening of *C. gigas* has been specifically related in the literature to the presence of the antifouling agent TBT, at levels as low as 0.05 µg/L in seawater (Alzieu et al., 1986; De Fur et al., 1999; Waldock and Thain, 1983). The decrease in the tissue yield is also considered to be a characteristic symptom of TBT exposure, where excessive weight gain mainly occurs in the shell (De Fur et al., 1999). The adverse effects of TBT on *C. gigas* were frequently observed in the 1980's, in various aquaculture sites in France and the UK (De Fur et al., 1999). In a previous study, Basheer et al. (2002) showed that TBT ranged from 0.43 to 3.2 µg/L in seawater around Singapore in 2000, i.e. well above the threshold limit of 0.05 µg/L presented by Alzieu et al. (1986). However, the

concentrations of TBT in the soft tissues of oysters in the present study were similar at both sites, and do not explain the shell differences at the two aquaculture sites. The levels of TBT in oysters (~500 ng/g dw; i.e. ~300 ng/g Sn dw) correspond to the middle range of concentrations in Korea (Shim et al., 1998).

VII – 4 – 3 Transplantation from 'contaminated' to 'clean' site

Levels of chlordanes in oyster tissues decreased by 99%, 97% for DDTs, >70% for PCBs and >90% for PBDEs after 33 days following transplantation from RSYC to P. Tekong. Therefore, the biological half-life of these contaminants in *C. gigas* cultured in Singapore is less than 33 days. In a laboratory experiment, the biological half-life of contaminants in the American oyster (*C. virginica*) reached 26-107 days for PCBs (Gardinali et al., 2004), but no data could be found in the literature for organochlorine pesticides in oysters. Depuration rates of POPs in oysters cultured in the tropical region appears to be faster or in the range of what has been reported from temperate latitudes, and this is reflected in the high growth rates achievable in warm tropical waters and, presumably, faster metabolic rates.

There are numerous scientific experiments that have investigated the effects of transplantation of marine organisms from uncontaminated to contaminated sites, but reports of the reverse are rare. Some work has been conducted on marine organisms to evaluate recovery from stress such as starvation and physical changes in the water (salinity or temperature changes) (e.g. Moran and Manahan, 2004). However, information on the reversibility of effects induced by anthropogenic contaminant bioaccumulation is lacking (Mc Dowell Capuzzo, 1996). A few studies have investigated the depuration of contaminants

such as POPs (Gardinali et al., 2004) and heavy metals (Chan et al., 1999), but biological parameters for oysters, including size and growth rates, were not assessed. The present field experiment shows that Pacific oysters can successfully recover from the adverse effects of water quality and bioaccumulation of POPs. Oysters transferred to a 'cleaner' area can recover in term of growth, tissue yield, shape and pollutant load, even after enduring several months of stress during early stages of their life cycle. Natural depuration (via transplantation of contaminated oysters before they are sold) could be used as a technique for decreasing risk associated with POP consumption of cultured oysters.

VII – 5 Conclusion

The experiment has revealed that pollution can have potentially adverse effects for the oyster aquaculture industry in Singapore. In particular, POPs and TBT represent a specific threat to both the yield and quality of oyster tissues. On a positive note, the effects of pollution on oysters have been found to be reversible, where transplantation to a 'clean' site will allow the organism to recover in terms of growth rate and tissue quality.

CHAPTER VIII – TRANSFER OF DDT IN AQUACULTURE SEABASS

VIII – 1 Introduction

The dynamics of POPs in the environment of temperate and polar regions has been well documented; however there is lack of data for aquatic systems in tropical areas. Recent studies of aquaculture systems in Europe and North America have revealed the presence of POPs, such as DDT, PCBs and PBDEs in fish meal (Antunes and Gil, 2004; Easton et al., 2002). The potential health risk to humans from exposure to POPs via the consumption of aquacultured seafood, such as salmon, has been recently reported (Hites et al., 2004). In 2001, Asia represented more than 80% of global aquaculture production (FAO, 2003); however, there is a paucity of data on the occurrence of POPs in cultured seafood in this region.

The Asian seabass, *Lates calcarifer*, is a carnivorous fish at the top of the marine food chain in tropical waters (Figure VIII-1). World production of cultured Asian seabass totalled 103,388 tons in 2001 (FAO, 2001), and the species is cultured throughout the Asia-Pacific region. Seabass is particularly susceptible to the bioaccumulation of POPs and has been used as an indicator species of marine contamination in both Europe and America (Jabber et al., 2001), but no records exist for Asia.

Figure VIII-1: The Asian seabass, *Later calcarifer*.

Although dietary exposure of fish to DDT has been documented in the past (Mitchell et al., 1977; Macek et al., 1970), the fish meal used in those ingestion studies was dosed at high levels – in the μg/g range which is not representative of prevailing exposure levels. More recently, Dabrowska et al. (1998) revealed that the uptake efficiency of PCB in fish at those ingestion dosages was lower than in fish exposed to dosages in the ng/g range.

The objective of this study was to determine the bioaccumulation and metabolism processes of DDT in Asian seabass when exposed to DDT contaminated feed in the ng/g range under controlled aquaculture conditions. DDT was selected as the compound of interest due to its continued usage in South East Asia and potential risk to the human food chain via seafood consumption.

VIII – 2 Materials and Methods

VIII – 2 – 1 Fish, feed pellets and *p,p'*-DDT

A total of 120 Asian seabass were purchased from San Lay Marine Culture Co Pte Ltd, Singapore. Mean fish weight was 460± 40 g at the start of the experiment. Feed pellets were obtained from Charoen Pokphands Foods Ltd (Muang Samutsakorn, Thailand). Pellet ingredients included fish meal, soy bean meal, rice bran, corn, broken rice, vitamins and minerals. *p,p'*-DDT was obtained from Aldrich (St. Louis, MO, USA) and was of 98% purity.

The successful spiking of the feed pellets was a key requirement to enable the accurate measurement of DDT bioaccumulation in the cultured seabass. Pellets were first checked to ensure that they did not dissolve in ethanol and remained intact in sea water after treatment. Pellets were then spiked with DDT as follows: 400 g of pellets were immersed in 400 mL of ethanol (95% denatured with methanol 5%). The ethanol pellet mix was then spiked with a solution of DDT dissolved in nonane (Merck, Darmstadt, Germany) and further diluted in ethanol to achieve target levels of 15 and 70 ng/g i.e. low dose (LD) and high dose (HD) respectively. A similar procedure was repeated with ethanol only (i.e. without DDT) for preparing blank feed pellets for the control batch of fish. Following spiking and drying by rotary evaporation to remove residual ethanol, the dry pellets were stored in a glass bottle in the dark at 4°C prior to use. Feed pellets were analyzed for *p,p'*-DDT, and its metabolites *p,p'*-DDD and *p,p'*-DDE. Target levels in the fish pellets were achieved with 0.44±0.03, 12.1±1.8 and 69.5±2.2 ng/g of *p,p'*-DDT in the control, LD and HD treatments respectively.

Traces of *p,p'*-DDD and *p,p'*-DDE were detected in the initial fish food, which are likely to have been metabolic by-products of the fish used in the fish meal. Calculations of dietary uptake efficiency and metabolism of *p,p'*-DDT were based on actual concentrations measured in the diet.

VIII – 2 – 2 Experimental design

The experiment was conducted at the Tropical Marine Science Institute (TMSI), Singapore using aquaculture research facilities. The seawater used for the experiment was pumped from a seawater intake 200 m offshore and purified via sand filtration before use in 4000 L aquaculture tanks which were equipped with a double drainage system to remove both floating and sinking organic waste. The water was exchanged at a continuous rate of 2 L per second. The illumination cycle was set at 12 hr light / dark. Seawater salinity was 31‰, temperature 29.6°C, pH=8.1 and the dissolved oxygen content was 6.2 mg/L throughout the duration of the experiment.

All fish used for the experiment were subject to initial acclimation for 24 days in a 4000 L tank and fed non-spiked pellets. During this period there were three fish fatalities out of a total of 120 fish, and no further fatalities occurred throughout the subsequent 42 day exposure period. After acclimation, the remaining fish were divided into three separate 4000 L tanks (i.e. 39 fish per tank) for pellet feeding with either control, LD or HD DDT spiked pellets. The fish were fed twice a day at 09.30 and 16.30 hrs by throwing small amounts of pellets into the tanks until all fishes were satiated i.e. until all individuals stopped feeding.

The exact weight of pellets used was recorded for each feeding event. Fish care included tank cleaning twice a week to remove residual solid waste.

VIII – 2 – 3 Sampling procedure

Prior to fish sampling from each of the three treatment tanks, fish were starved for two days to ensure that the digestive system was cleared of any ingested pellet material. Every 7 days for 42 days, three fish from each tank were randomly removed for analysis. Upon sampling, fish were placed into freezing seawater to slow fish metabolism and induce death. The weight and length for each individual was then recorded prior to dissection. The right muscle fillet, liver and visceral fat surrounding the intestines was collected and weighed. The liver comprises two red lobes well separated from the rest of the internal organs and therefore easily identified for removal (Figure VIII-2). After completion of the experiment, i.e. after 42 days of pellet ingestion exposure, the brain and the remaining tissues (i.e. bones, scales, head and any remaining organ tissue) were also collected for analysis from the remaining individuals. Samples of each tissue type from the three individuals sampled for each treatment, throughout the experiment, were mixed in a stainless steel blender to form a composite sample. Tissue samples were stored in glass bottles and kept in darkness at minus 20°C prior to analysis. Lipid content in the fish tissues was determined gravimetrically following Soxhlet extraction (See Section III-4-5, Chapter III).

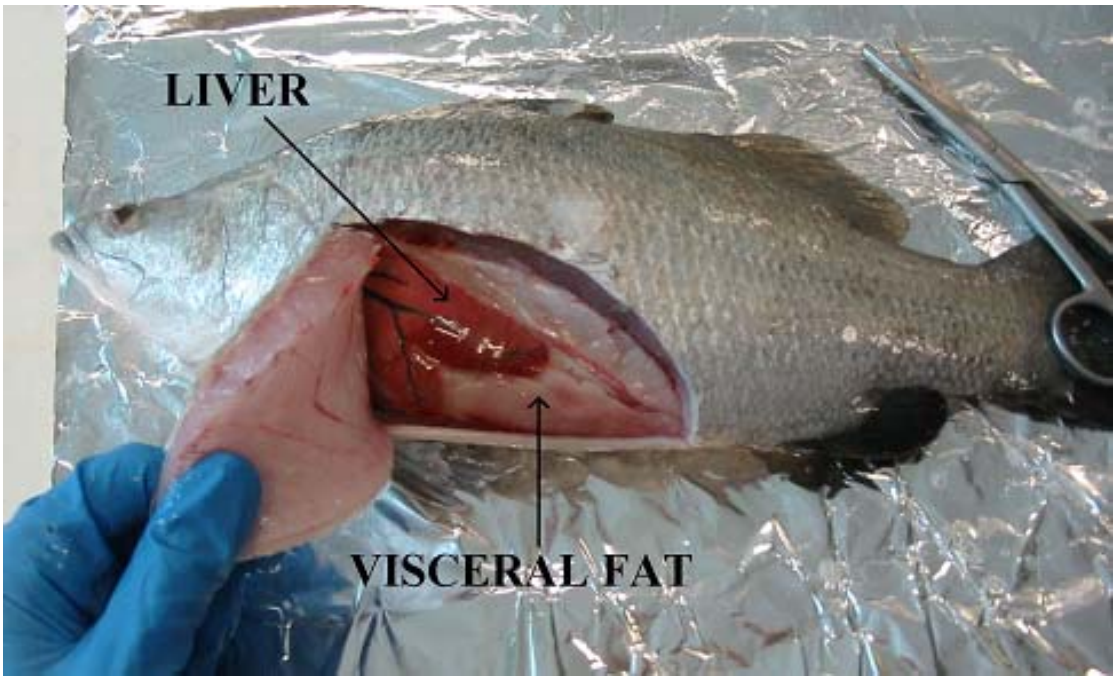

Figure VIII-2: Dissection of a seabass showing the position of the liver and the visceral fat.

One liter of sea water was sampled at the inlet for each tank to determine background DDT exposure levels to the fish at each sampling episode. Water samples were analyzed for *p,p'*-DDT, and its metabolites *p,p'*-DDD and *p,p'*-DDE using conventional techniques (USEPA, 1984).

VIII – 2 – 4 Tissue analysis

Details on the extraction, cleanup and quantification of *p,p'*-DDT, and its metabolites *p,p'*-DDD and *p,p'*-DDE were presented in Section III-4 to III-6, Chapter III. The sample size for extraction was optimized based on the expected concentration in each tissue type. Details on

the amounts of tissue, the volume of n-pentane/DCM (1:1, v:v) and the MAE parameters used in analysis are given in Table VIII-1.

Tissue type	Lipid content (%)	Average organ final weight (all dosage) (g)	Sample size for extraction (g)	Extraction solvent volume (mL)	MAE max power (W/sample)
Muscle	4.9	275	3.0	18	112
Liver	38.5	12	2.0	12	135
Visceral fat	90.3	18	1.0	10	150
Brain	24.0	0.61	0.9	10	200
Remaining tissues	8.8	350	5.0	35	140

Table VIII-1: MAE parameters used for each fish tissue types.

VIII – 2 – 5 Quality assurance

MDLs for each analyte were determined by quantification of five blank extracts. The MDL values were calculated for *p,p*'-DDT, *p,p*'-DDD and *p,p*'-DDE in each tissue type and were typically lower than 0.2 ng/g ww in fat and brain tissue, and lower than 0.1 ng/g ww in the other tissues. Surrogate standards were satisfactorily recovered with an average recovery of 104%±6% for PCB 61 and 105%±7% for PCB 55. Recoveries were identical for low-lipid content tissues such as muscle (4.9% lipid content), as well as for high-lipid content tissues such as liver (39% lipid content) and visceral fat (90% lipid content). To determine analytical

reproducibility, four replicates were analyzed from homogenates of separate muscle, liver and visceral fat tissue samples. RSD for the four replicates ranged from 3.2 to 8.7% according to the pesticide analyte, indicating that acceptable homogenization was achieved. The standard reference material, SRM2978 was analyzed to validate the complete analytical method. Certified values were recovered with an average of 117%±1% for *p,p'*-DDT, 90%±3% for *p,p'*-DDD; 109%±8% for *p,p'*-DDE.

p,p'-DDT, *p,p'*-DDE or *p,p'*-DDD were not detected in the seawater used for the experiment. The MDL for *p,p'*-DDT and its metabolites in the water sample was 0.02 ng/L. As *p,p'*-DDT and its metabolites were not detected in seawater, it may be assumed that all DDT detected in the fish were derived from the ingestion of pellets.

VIII – 3 Results

V – 3 – 1 Tissue partitioning of *p,p'*-DDT and its metabolites

After completion of the experiment, i.e. after 42 days of exposure, levels of *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE were quantified in the muscle, liver, visceral fat, brain and the remainder of the fish for each ingestion exposure level (See Table VIII-2).

	Tissue	*p,p'*-DDT	*p,p'*-DDE	*p,p'*-DDD
Control	Muscle	0.21	0.90	0.19
	Liver	2.0	8.9	7.1
	Visceral fat	14	37	26
	Brain	0.71	4.6	4.5
	Remaining tissues	1.0	2.4	2.0
LD	Muscle	2.1	1.0	0.26
	Liver	11	13	16
	Visceral fat	65	34	30
	Brain	4.5	5.5	5.3
	Remaining tissues	2.5	1.1	1.2
HD	Muscle	6.6	0.84	1.0
	Liver	19	7.3	34
	Visceral fat	270	32	42
	Brain	33	11	29
	Remaining tissues	19	3.7	2.6

Table VIII-2: Individual organ concentrations (ng/g ww) in control and exposed *L. calcarifer* after 42 days.

The relative partitioning of DDT compounds between the seabass tissues, expressed as a percentage of the total concentration present in the whole fish, for each DDT ingestion exposure level at the end of the 42 days, is shown in Figure VIII-3. The partitioning, in terms of mean percentage levels, for *p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD between the tissue types of the control, low dose and high dose exposed seabass was : 14.8% ± 7.5% in muscle tissue; 3.5% ± 1.9% in the liver, 37.1% ± 8.7% in visceral fat; 0.11% ± 0.03% in brain; and 45.5% ± 4.1% in the remaining fish tissues.

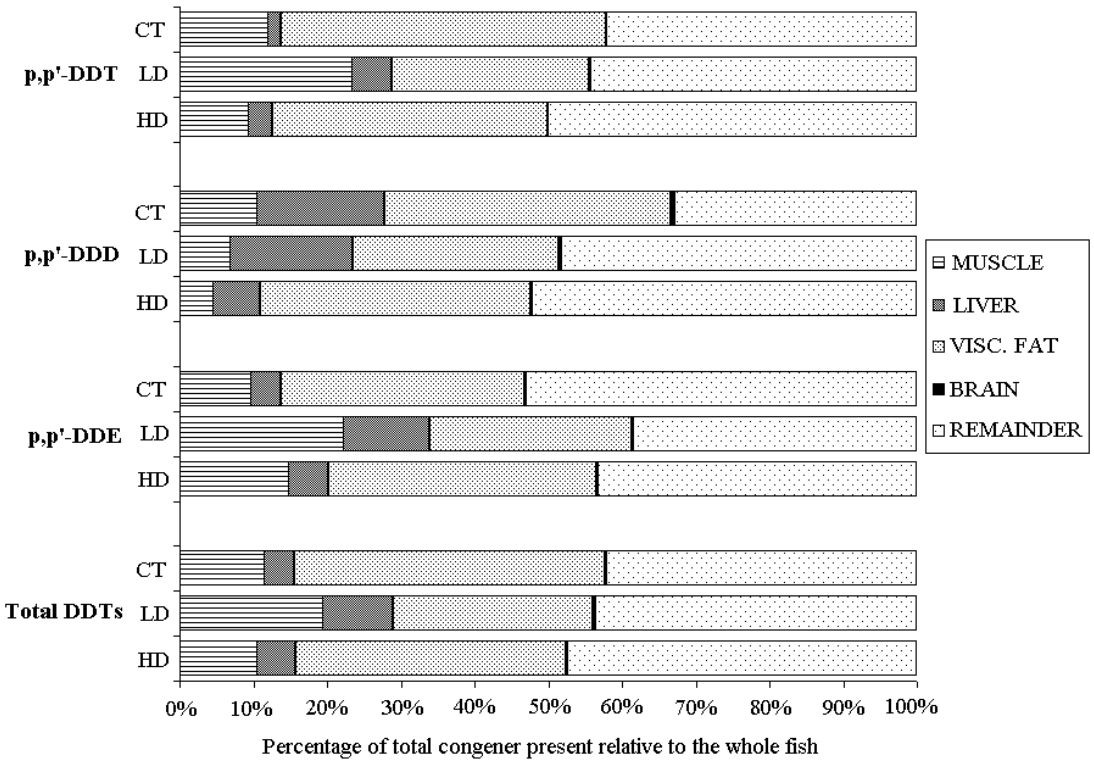

Figure VIII-3: Relative partitioning of *p,p'*-DDT and its metabolites in *L. calcarifer* tissue types (CT: control, LD:low dose, HD: high dose).

It is broadly acknowledged that lipid contents are associated with higher levels of POPs (Addison, 1982), leading to lipid normalization of organ concentrations in reported data. However, the partitioning of DDT or other POPs is not entirely definitive and can vary according to fish species. The concentrations of pesticide versus tissue lipid content are shown in Figure VIII-4 for seabass exposed to the highest level of DDT ingestion. In the muscle and brain tissue, as well as the visceral fat and remaining tissues of the fish, the total DDT concentration is specifically and significantly linearly correlated with lipid content, where r^2=0.99. In contrast, the DDT concentration in liver tissue is an outlier and does not fit this relationship. The same linear relationship can also noted for the control fish, where the lipid content is also significantly correlated with concentrations of *p,p'*-DDT (r^2=0.91), *p,p'*-

DDD (r^2=0.98), *p,p'*-DDE (r^2=0.96). However, due to the lack of data for lipid content in the 30-90% range, the linear correlation should be further confirmed.

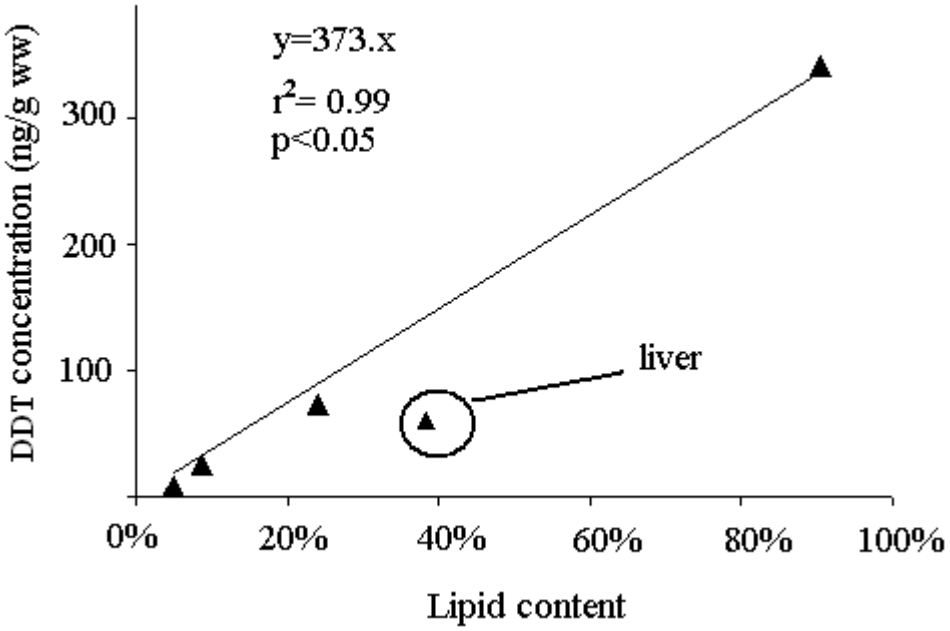

Figure VIII-4: Concentration of total DDT (sum of *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD) versus the lipid content in *L calcarifer* tissues.

VIII – 3 – 2 Dietary uptake efficiency of *p,p'*-DDT in seabass

The concentrations of DDT and its metabolites were measured in muscles, liver and visceral fat tissues for the control, LD and HD exposed fishes. Figures VIII-5a to c show concentrations over the duration of the 42 day experiment for the three tissues types of the HD exposed seabass. Concentrations of *p,p'*-DDT in the muscle tissues and the visceral fat of the HD exposed fish increased linearly with time, but concentrations of *p,p'*-DDD and *p,p'*-DDE remained constant. In the liver of the HD exposed fish, an increase of *p,p'*-DDD was observed, but *p,p'*-DDT and *p,p'*-DDE showed no notable accumulation. Observations were similar for the LD exposed fish, and levels of *p,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE in the control fish tissues remained constant.

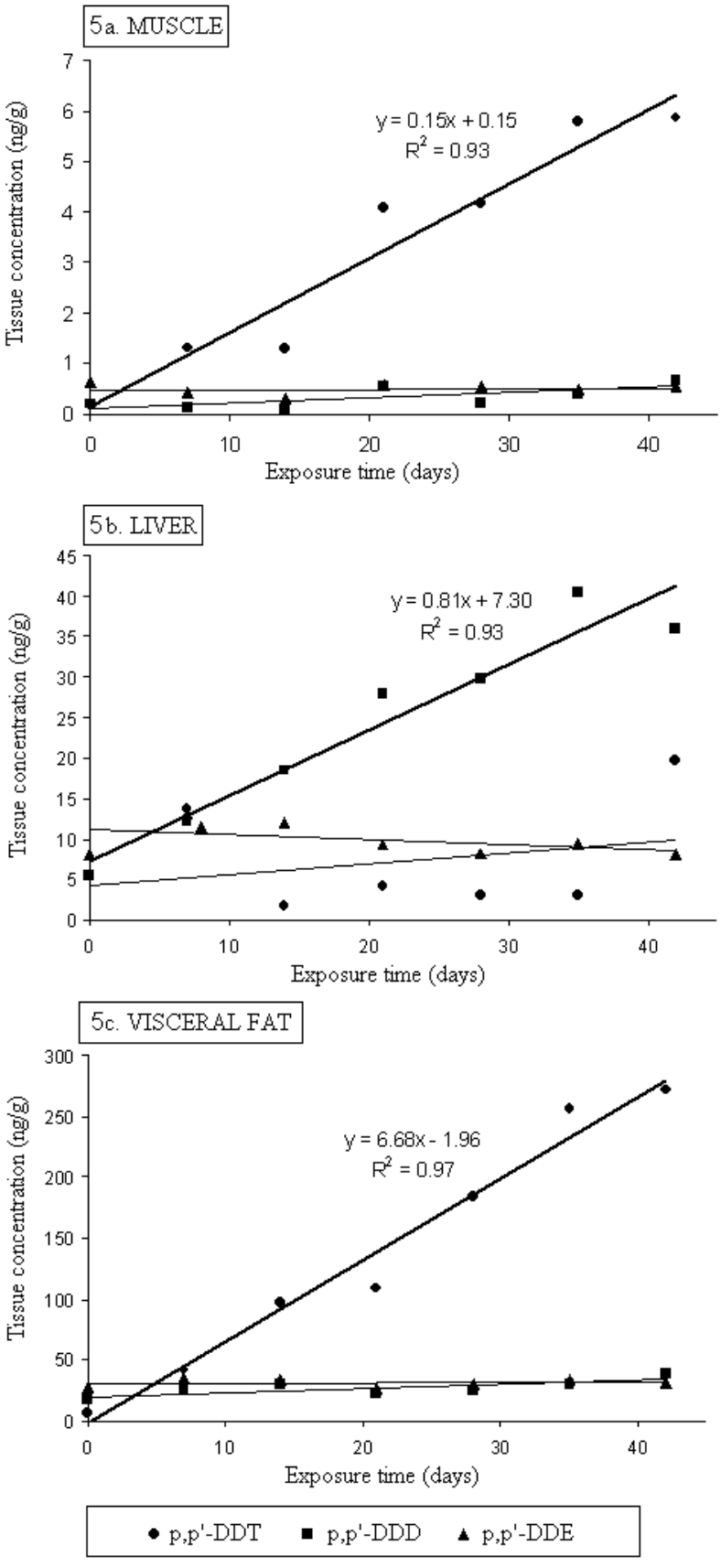

Figure VIII-5

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Figure VIII-5: Concentrations of *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD in the three tissue types of the high dose exposed seabass (5a: muscle tissues, 5b: liver, 5c: visceral fat). The linear correlation coefficients are given for *p,p'*-DDT in the muscle tissues and the visceral fat, and for *p,p'*-DDD in the liver.

To summarize, the coefficients of the linear correlations for the concentration of *p,p'*-DDT and its metabolites versus time are presented in Table VIII-3, for each treatment and each tissue type. The coefficients represent the bioaccumulation rates (i.e. µg/kg of tissue/day) in the three tissues types. Negative values in Table VIII-3 mean that the growth rate of the seabass exceeded the accumulation rate of the compound via ingestion exposure. This phenomenon is known as 'growth dilution' (Gobas, 1993).

		Accumulation rate (µg/kg/day)		
	Compound	**Muscle**	**Liver**	**Visceral fat**
Control	*p,p'*-DDD	n.a.	0.017	-0.087
	p,p'-DDE	-0.003	0.074	-0.12
	p,p'-DDT	n.a.	0.003	-0.067
LD	*p,p'*-DDD	n.a.	0.16	-0.006
	p,p'-DDE	0.004	-0.020	-0.16
	p,p'-DDT	0.055	n.a.	1.3
HD	*p,p'*-DDD	0.011	0.81	0.33
	p,p'-DDE	0.001	-0.054	0.023
	p,p'-DDT	0.15	0.13	6.4

Table VIII-3: *p,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE bioaccumulation coefficients. Accumulation rates were derived from the tissue concentration versus the time regression correlation and are expressed in µg/kg/day (n.a. = not applicable, below detection limit).

For muscle tissue, the linear regression slope for the HD exposed seabass (i.e. 0.15 µg of *p,p'*-DDT/kg of muscle tissue/day) was approximately three times greater than that for LD exposed fish (i.e. 0.055 µg of *p,p'*-DDT/kg of visceral fat/day). For visceral fat, the linear regression slopes were five times greater for HD compared to LD exposed fish (i.e. 6.4 µg versus 1.3 µg of *p,p'*-DDT/kg of visceral fat tissue/day). These figures approximate to the proportional relationship of the concentration of *p,p'*-DDT in fish pellets between the two exposure levels. The bioaccumulation of *p,p'*-DDT in the muscle tissue and visceral fat appeared to be proportional to the level of pesticide exposure.

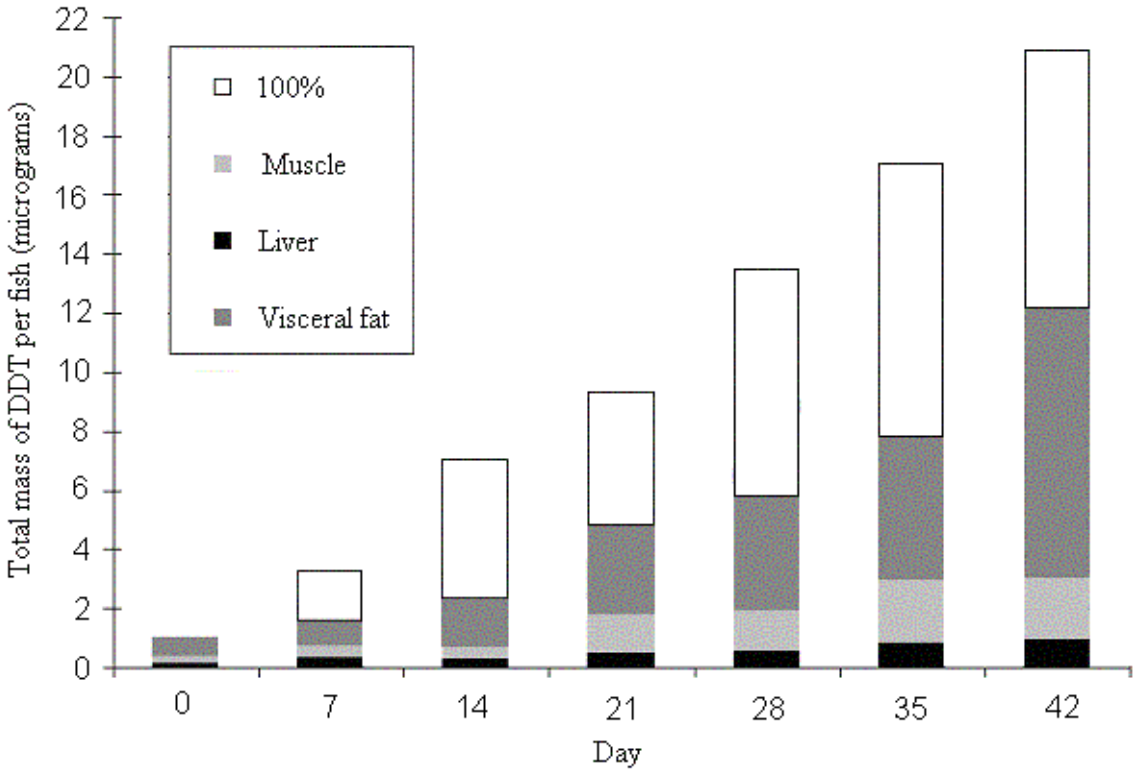

Figure VIII-6: Bioaccumulation of total DDT in the muscle, liver and visceral fat tissues for the high dose exposed seabass, relative to total amount of DDT ingested from the food over the 42 day exposure period. '100%' refers to the total amount of DDT contained in the feed pellets multiplied by the food intake per fish.

Figure VIII-6 shows the total bioaccumulation of DDT i.e (*p,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE) in the HD exposed fish for the three tissue types studied. In all tissue types, the amount of pesticide increased over the duration of the experiment. The segment of the graph bar shown in white on Figure VIII-5 represents the difference between the total amount of DDT contained in the feed pellets multiplied by the food intake per fish and the total concentration of all DDT compounds detected in each of the three tissue types (i.e. 100% intake of *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD contained in the food). By adding the three tissue concentrations of the HD exposed seabass together for each weekly sample period and plotting it against the total amount of DDT ingested from the pellets, the slope of a linear regression (r^2=0.94) gives the % of DDT ingested that is retained in these tissue types. This percentage equates to 53% total retention at the end of the experiment. This figure is very close to the sum of average percentage in muscles, liver and visceral fat determined analytically (55.4%). Therefore, most of the unaccounted 47% of DDT ingested by the seabass can be attributed to its accumulation in the remainder of the fish. The dietary uptake efficiency in the whole fish may be estimated at 98% in this study.

VIII – 3 – 3 DDT metabolism in exposed seabass

It is important to determine whether metabolism of DDT occurred in the cultured seabass, or whether the presence of *p,p'*-DDD and *p,p'*-DDE was due to these metabolites being ingested from the feed pellets. The levels of *p,p'*-DDD and *p,p'*-DDE, for the LD exposed and control fishes are too close to the analytical detection limit to determine the possibility of metabolic transformation, but a calculation of the rate of metabolism is possible for the HD

exposed seabass. The mass balance of *p,p'*-DDE and *p,p'*-DDD in the whole body of the HD exposed fish at the end of the experiment is summarized in Figure VIII-7.

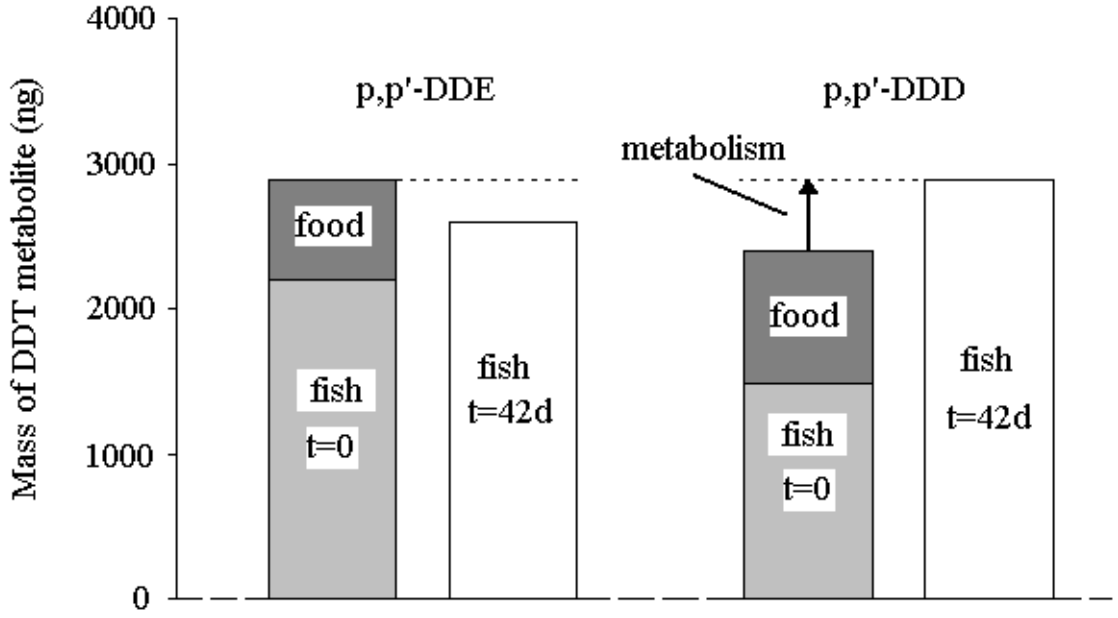

Figure VIII-7: Mass balance of *p,p'*-DDE and *p,p'*-DDD in the high dose exposed fish. Amounts of metabolites in the fish at the beginning (t=0) and the end (t=42 days) are compared taking into account ingestion from food.

From the present calculations, the increase of *p,p'*-DDD in seabass during the experiment was higher than could have been theoretically bioaccumulated at a 100% intake rate from the ingested pellets. As the water was free of DDT compounds, this surplus must have been derived from *p,p'*-DDT metabolized within the fish itself. However, no increase in *p,p'*-DDE levels in the tissues analyzed was apparent. By comparing the excess of *p,p'*-DDD to the mass of *p,p'*-DDT ingested by the HD exposed fish over the 42 days duration of the experiment, only 2.5% of the *p,p'*-DDT ingested was actually metabolized.

VIII – 4 Discussion

VIII – 4 – 1 Bioaccumulation processes following exposure at ng/g levels

Dabrowska et al. (1998) suggested a reconsideration of PCB uptake efficiency values, as it was shown that rainbow trout had a higher uptake rate at a ng/g, rather than a μg/g, ingestion level, which is more environmentally relevant. In this study, the partitioning, uptake efficiency and metabolism of *p,p'*-DDT in the Asian seabass was evaluated.

Previous studies on dietary exposure to DDT in fish have reported that accumulation occurs primarily in the liver, at levels of up to 95% of the total body burden of ingested DDT (Mitchell et al., 1977; Dvorchik and Maren, 1972). However, these conclusions were derived for a single dosage at μg/g levels via food ingestion and intravenous injection and may not be representative of prevailing DDT concentrations in environmental and aquaculture systems (Antunes and Gil, 2004). In contrast, the present data indicate that, at a more realistic ingestion exposure dosage, in the ng/g range in fish meal, the partitioning of DDT is more prevalent throughout the body tissues of the fish, where the liver only accounts for a minor fraction of the overall body burden. Mitchell et al. (1977) reported that the distribution of DDT in the tissues of cod following dietary exposure was not proportional to tissue lipid content. *p,p'*-DDT and its metabolites, following dietary exposure at ng/g levels were partitioned in *L. calcarifer* according to the tissue lipid content. Unrealistically high exposure dosages to the liver may obscure or negatively impact the enzyme metabolism process, thereby resulting in apparently high liver bioaccumulation rates of DDT, as reported in earlier studies (Mitchell et al., 1977; Dvorchik and Maren, 1972).

Uptake of POPs in fish is characterized by two phases: an initial phase with a rapid linear uptake following ingestion exposure to the contaminant, followed by a secondary phase characterized by minimal uptake referred to as 'steady-state' (Macek et al., 1970). This study shows that cultured seabass exposed to *p,p'*-DDT at a ng/g level in feed pellets were still in the initial phase of contaminant uptake upon reaching market size (i.e 700 g), and that dietary uptake efficiency of *p,p'*-DDT in the whole seabass was 98% during this phase. Previous studies on dietary uptake for *p,p'*-DDT in fish report lower values ranging from 20-64% in rainbow trout (Macek et al., 1970; Gobas et al., 1988), and 14-71% in cod (Mitchell et al., 1977). In these cases, uptake efficiencies were investigated at μg/g levels in the food. The present data also support a reconsideration of the uptake efficiency value for *p,p'*-DDT in fish following ingestion of food at the more realistic ng/g level.

By comparing the excess of *p,p'*-DDD to the mass of *p,p'*-DDT ingested by the HD exposed fish over the 42 days duration of the experiment, only 2.5% of the *p,p'*-DDT ingested was actually metabolized to *p,p'*-DDD. Kitamura *et al.* (1999) explained that *p,p'*-DDT is converted by a reductive dechlorination process in the liver to *p,p'*-DDD by induced hepatic microsomal enzymes (cytochrome P450), where dechlorination is the first and rate limiting step in *p,p'*-DDT transformation. The biodegradation of *p,p'*-DDD leads to the formation *p,p'*-DDE. However, in this study, this reaction could not have occurred as no increase in *p,p'*-DDE levels in the tissues analyzed was apparent. Previously, Serrano et al. (2003) reported levels of *p,p'*-DDT and its metabolites in both fish feed and cultured European seabass (*Dicentrarchus Labrax*) after 6 and 24 months of fish ingestion exposure. The ratio of *p,p'*-DDT to its metabolites did not differ significantly in the feed and in the fish, thereby

indicating that the European seabass also does not show notable biotransformation of *p,p'*-DDT to *p,p'*-DDE.

VIII – 4 – 2 Risk assessment for aquaculture

Edible parts of the fish, i.e. the muscle tissue (fillet), typically contained less than 25% of the total DDT body burden in the cultivated seabass. The amount of analyte measured in the remainder of the fish (including head, bones, skin and other organs) is due to the proportional weight of these organs i.e. >50% of the total mass of the fish. However, it is important to consider the pesticide burden in the non-edible portion of the fish as this is frequently processed for fish meal as the tissues are rich in proteins and lipids (Boonyaratpalin, 1997). Therefore, POPs in these tissues may be readily re-introduced into the food chain. Evidence has recently been published linking the body burden of POPs in salmon to the ingestion of contaminated fish meal under intensive aquaculture conditions (Easton et al, 2002).

In terms of fish toxicology, the distribution of *p,p'*-DDT throughout the tissues of the Asian seabass analyzed, indicates that all organs are potentially at risk when fish are exposed to the pesticide. It is known that the DDT and its metabolites in fish are potentially hepatocarcinogenic (Chang et al., 1998). Because of its higher lipid content, DDT levels in brain tissue samples were one order of magnitude higher than in muscle tissue. Previously, *p,p'*-DDT was reported to be higher in the brain of freshwater fish species compared to muscle tissues (Kiziewicz and Czeczuga, 2003), but no comparative data are available for cultured marine species. The *p,p'*-DDD concentration in the brain tissues of the seabass increased with the DDT ingestion exposure level in the food. Thus, the brain is an organ that

has a propensity to accumulate organochlorine pesticides following ingestion. As lipophilic compounds, such as DDT, have a greater ability to absorb in the blood and penetrate cell membranes (Sipes and Gandolfi, 1991; Nims et al., 1998) it is therefore likely that POPs reach cerebral tissues via transport in the blood. Other POPs, such as PCBs, have also been reported to be unaffected by the blood-brain barrier which is claimed to protect the brain from exposure to various xenobiotics (Tampal et al., 2003). The bioaccumulation of DDT (and its metabolites) in the brain is known to be a source of biochemical damage, including inhibition on ATP based reactions (Davis and Wedemeyer, 1971), hepatic enzyme induction (Nims et al., 1998) and disruption of hormonal mechanisms (Gonzáles and Piferrer, 2003).

VIII – 5 Conclusion

The primary objective, i.e. to evaluate ingestion exposure of Asian seabass in a simulated aquaculture system, has been achieved. Results show that bioaccumulation mechanisms following ingestion exposure to *p,p'*-DDT at environmentally relevant levels (ng/g range in fish meal) are different from the unrealistic dosage levels used in previous environmental modeling studies. As a consequence, the continual introduction of POPs, such as *p,p'*-DDT, into aquaculture systems via contaminated fish meals is likely to result in a substantial increase of the pollutants in the fish portions destined for human consumption. It is therefore advisable to control the levels of contaminants in fish meals used in the aquaculture industry.

CHAPTER IX – POPs IN TYPICAL SEAFOODS CONSUMED IN SINGAPORE

IX – 1 Introduction

Seafood consumption is a rich source of vitamins, minerals, proteins and Omega-3 polyunsaturated fatty acids that have a wide range of beneficial effects for human health (Sidhu, 2003). However, seafood is also recognized as a major source of heavy metal exposure (Nasreddine and Parent-Massin, 2002) and POPs (Simmonds et al., 2002). Episodes of food poisoning have been reported following consumption of seafood contaminated with pollutants such as mercury (Gochfeld, 2003). Chronic exposure to heavy metals and POPs may also result in long term adverse health hazards (Smith and Gandolli, 2002; De Wit, 2002). Reports of POPs in seafood include xenobiotics such as PCBs (Bjerregard et al., 2001), organochlorine pesticides (Smith and Gandolli, 2002), PBDEs (Bocio et al., 2003), dioxins and furans (Schecter et al., 2003). In particular, the impact of consuming farmed fish has recently raised health concerns as elevated levels of POPs have been measured in edible tissues relative to wild fish (Antunes and Gil, 2004; Easton et al., 2002; Hites et al., 2004).

In the last decade, the intake of POPs from food in the developing countries of Asia has been estimated to be between 5 to 100 times higher than in industrialized countries (Kannan et al., 1997). However, although literature data exist on POP levels in fish (Schecter et al., 2003), no information is available regarding the human intake of contaminants via seafood consumption in Asian countries, including Singapore. Seafood consumption in Singapore

averaged 46.3 and 49.9 g/day for women and men respectively in 1998 (Ministry of Health, Singapore, 2001), which is comparable to typical seafood consumption rates in Taiwan (Chien et al., 2003), but more than twice the intake in San Francisco, USA (Greenfield et al., 2003). Most seafood consumed in Singapore is imported - principally from elsewhere in Asia (e.g. prawns from Thailand), but also from Europe (e.g. salmon from Norway) and the Americas (e.g. scallops from USA), (Singapore Trade Development Board, 2001).

In this study, the levels of POPs (i.e. PCBs, PBDEs and organochlorine pesticides) were measured in the edible portions of twenty different seafood types commonly consumed in Singapore. This is the first report of a contaminant risk assessment based on seafood consumption in South-east Asia.

IX – 2 Materials and Methods

IX – 2 – 1 Sample collection and preparation

Twenty types of seafood were collected from local supermarkets between June 2002 and June 2003. Details of the samples analysed are presented in Table IX-1. The selection of the seafood types was based on supermarket sales figures and represents typical consumption patterns in Singapore (personal communication). Samples were purchased in their usual packaging. In the laboratory, the edible portions of the samples were homogenized in a stainless steel blender prior to analysis.

Seafood type	Species	Sample size
shark steak	species not identifiable	4 fillets
shark fin	species not identifiable	2 x 100 g
cod fillet	species not identifiable	2 fillets
stingray fillet	*Dasyatis kuhlii*	2 fillets
tuna steak	species not identifiable	4 fillets
canned tuna	species not identifiable	2 cans
silver pomfret fillet	*Pampus argenteus*	3 fish
selar fillet	*Selar crumenophtalamus*	3 fish*
kuning fillet	*Selaroides leptolepis*	10 fish*
conger eel fillet	species not identifiable	2 fillets
greasy grouper fillet	*Epinephelus coioides*	3 fish
sea bass fillet	*Lates calcarifer*	3 fish
song fish fillet	*Aristichthys nobilis*	2 fillets*
salmon fillet	*Salmo salar*	3 fillets
squid ring	species not identifiable	140 g*
grey prawn	species not identifiable	12 prawns*
giant tiger prawn	*Penaeus monodon*	12 prawns*
flower crab	*Portunus pelagicus*	3 crabs*
green mussel	*Perna viridis*	9 mussels*
scallop	*Pectinidae spp*	10 scallops*

Table IX-1: Sample types and characteristic of seafood. * refers to a pooled sample.

IX – 2 – 2 POPs analysis

The extraction of POPs using MAE and their quantification has been presented and validated in Chapter IV. PBDE congeners 47, 99 and 100 were quantified in each sample. Samples were spiked with PCB 55 and 61 as recovery standards. The standard reference material SRM 2978 green mussel tissue was analyzed to validate the complete analytical method.

IX – 2 – 3 Risk assessment

Periodically, the Ministry of Health in Singapore conducts a survey on the dietary habits of the population. Latest available seafood consumption figures, from 1998 (Ministry of Health, Singapore, 2001) were used for risk assessment calculations using analytical data obtained from this study. In this survey, the mean daily intake (MDI) of fish/seafood for the general population of Singapore is similar for both females and males and approximated to 46 and 50 g/day, respectively. Therefore, an average MDI for seafood of 48 g/day was used in dietary exposure calculations, together with an average body weight of 60 kg. The estimated MDI of contaminants from seafood was calculated as the MDI of seafood multiplied by the mean concentration of contaminants in the twenty seafood types. Using the methodology specified by the US Environmental Protection Agency (Dougherty et al., 2000), the mean concentration of contaminants was calculated according to two hypotheses: 'non-detect samples are equal to zero' i.e contaminant values below the limit of analytical detection are ascribed a value of zero; and 'non-detect samples are equal to half of the limit of detection' i.e. contaminant values below the limit of analytical detection are ascribed a value of 50% of the limit of analytical detection. The first hypothesis tends to underestimate the concentration, and therefore the intake of contaminants.

IX – 3 Results

IX – 3 – 1 Quality assurance

Recoveries of POP surrogate standards were satisfactory with an average value of 102% ± 16%. The standard reference material SRM 2978 – green mussel tissue– was analyzed to validate the complete analytical method. The certified values for PCBs, DDTs and chlordanes were achieved with an average recovery of 100% ± 22%, with typically less than 30% difference between analytical and certified values, except for trichloro-PCBs. Analysis of POPs in seafood samples was duplicated.

IX – 3 – 2 POPs in the seafood

Levels of α- and γ-chlordane, DDTs, PCBs and PBDEs (sum of all congeners) in the edible parts of the 20 types of seafood analyzed are presented in Figures IX-1a, 1b, 1c and 1d. The average concentrations of POPs in seafood, as well as their limit of detection and relative occurrence amongst the seafood types are reported in Table IX-2 in Chapter IX-3-3. Chlordanes, DDTs and PCBs were detected in 75, 90 and 100% of the seafood types, respectively. On the contrary, mirex and PCNB were only detected in 10 and 15 % of the seafood types, at a concentration two orders of magnitude less than for DDTs and PCBs, respectively. Levels of chlordanes were below 1 ng/g ww in all seafood types, except for green mussels (14.4±2.0 ng/g ww) and salmon fillets (2.9±1.7 ng/g ww).

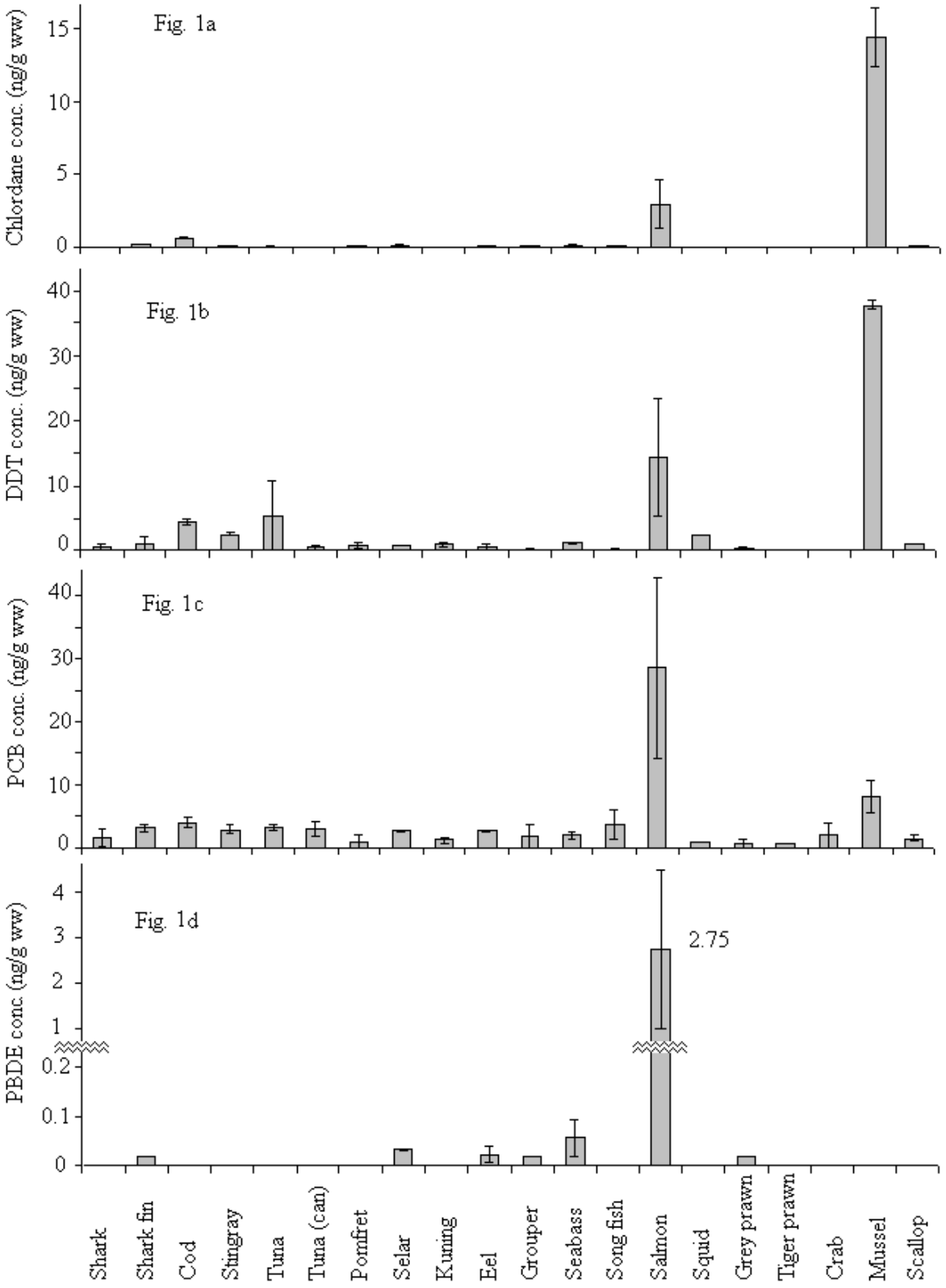

Figure IX-1: Total levels in ng/g wet weight (ww) of chlordanes (1a), DDTs (1b), PCBs (1c) and PBDEs (1d) in the major seafood types commonly consumed in Singapore (mean level±SD).

Levels of DDTs were below 5 ng/g ww, except for green mussels (37.8±0.7 ng/g ww) and salmon fillets (14.4±9.1 ng/g ww). Levels of PCBs were below 5 ng/g ww in all seafood types except for green mussels (8.2±2.6 ng/g ww) and salmon fillets (28.5±14.4 ng/g ww). Levels of PBDEs were below 0.1 ng/g ww for all seafood types, except salmon fillets (2.8±1.8 ng/g ww).

Principal component analysis of PCB congener profiles of seafood relative to commercial Aroclor mixtures is shown in Figure IX-2. PCB congener profiles for Aroclor mixtures 1221, 1232, 1242, 1248, 1254, 1260 and 1262.

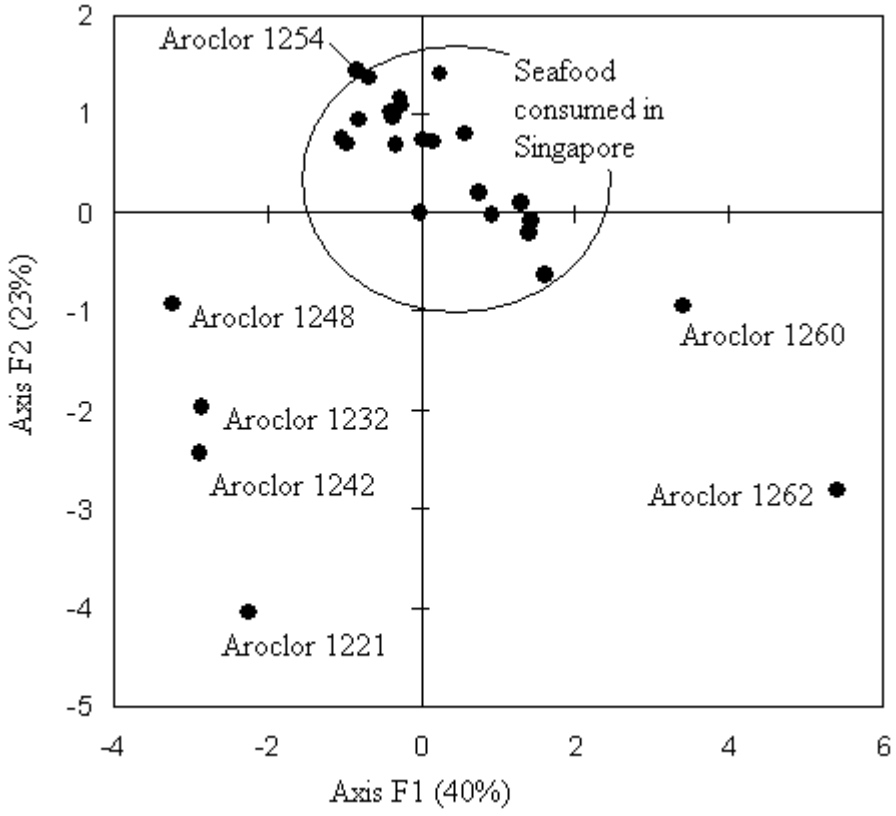

Figure IX-2: Bi-plots showing the first two principal components of relative individual polychlorinated biphenyl (PCB) congener profiles in seafood in relation to congener profiles for Aroclor mixtures 1221, 1232, 1242, 1248, 1254, 1260 and 1262.

The first principal component, F1, has a positive loading on hepta-CBs and octa-CBs and a negative loading on tri-CBs and tetra-CBs. The second principal component, F2, has a positive loading on penta-CBs and hexa-CBs and negative loading on tri-CBs, hepta-CBs and octa-CBs. The PCB congener profile of seafood types analysed reflects the presence of a mixture of Aroclor 1254 and Aroclor 1260 congeners, with the majority of the 20 types closely matching the PCB congener profile of Aroclor 1254.

IX – 3 – 3 Mean daily intake

MDIs of POPs in seafood types typically consumed in Singapore are presented in Table IX-3. MDIs were similar (difference <10%) regardless of the value attributed to the non-detect samples (i.e. zero or half of the detection limit), except for PBDEs, PCNB and mirex. Differences of greater than 10% can be attributed to the low occurrence of contaminants amongst food types (Dougherty et al. 2000). The mean daily intake of DDTs, PCBs and PBDEs from seafood for a 60 kg person in Singapore reaches 3.0, 3.0 and 0.1 ng/kg body weight per day respectively.

Contributions of specific types or groups of seafood to the MDI of POPs were calculated for the hypothesis of 'non-detect samples are equal to zero' (See Figures IX-3). On this basis, it can be concluded salmon consumption accounts for 19%, 38% and 94% of the mean calculated intake of DDTs, PCBs and PBDEs respectively, and green mussel consumption accounts for to 51%, 11%, and 77% of the mean calculated intake of DDTs, PCBs and chlordanes respectively.

Contaminant	MDL (ng/g ww)	Mean level (range)[1] (ng/g ww)	Percentage of seafood types with levels above MDL	Mean daily intake (ng/kg body weight/day)		Oral RfD [2]	Cancer [3] benchmark concentration
				Hypothesis no detect = 0	Hypothesis no detect=0.5 DL		
chlordanes [a]	0.04	0.95 (BLD-14.39)	75%	0.75	0.76	500	1
DDTs [b]	0.04 to 0.09 [c]	3.76 (BLD-37.84)	90%	3.00	3.01	500 as p,p'-DDT	3
PCNB	0.04	0.03 (BLD-0.13)	15%	0.01	0.02	3000	
heptachlor	0.08	0.39 (BLD-6.52)	35%	0.29	0.31	500	0.22
hHeptachlor epoxide	0.04	0.16 (BLD-2.13)	55%	0.123	0.130	13	
mirex	0.02	0.01 (BLD-0.04)	10%	0.003	0.012	200	
PCBs	0.01 to 0.2 [c]	3.72 (0.61-28.47)	100%	2.99	2.99	20 as Aroclor 1254	0.13
PBDEs	0.01 to 0.03 [c]	0.17 (BLD-2.75)	30%	0.117	0.134	2000	

Table IX-2: Mean level, occurrence and mean daily intake of POPs from seafood for a 60 kg person in Singapore. [a]. sum of α and γ-chlordanes; [b]. sum of *p,p*'-DDT, *p,p*'-DDD and *p,p*'-DDE; [c]. range for all congeners. [1] Mean concentration (range between brackets) amongst the various types of seafood for the hypothesis "Nondetect = 0.5 detection limit" - BLD: below limit of detection; [2]. Data obtained from USEPA' Integrated Risk Information System (http://www.epa.gov/iris); [3] represents the exposure concentration at which lifetime cancer risk is one in one million.

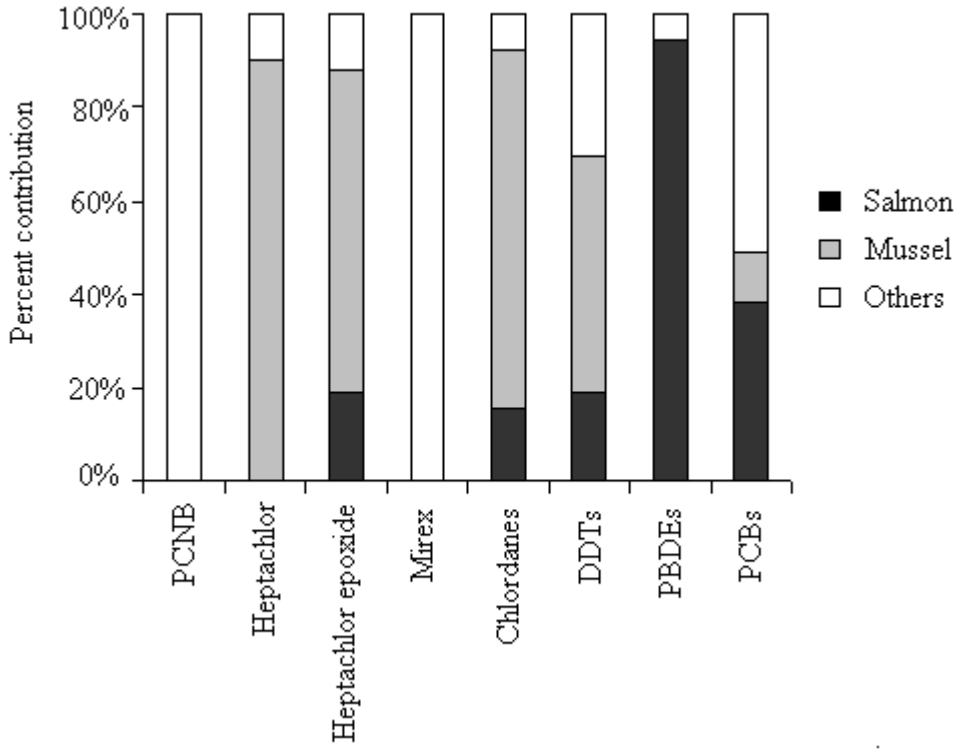

Figure IX-3: Percentage contribution of salmon, green mussels and other types of seafood to the mean daily intake of POPs via seafood consumption in Singapore.

IX – 4 Discussion

IX – 4 – 1 Comparison with international data

Amongst the 20 seafood types analyzed, green mussel and salmon fillets samples contained the highest levels of POPs. Green mussels are filter-feeders and therefore readily accumulate POPs. The levels found in market samples were in the upper range of concentrations found in wild mussels in South-east Asia (See Section II-3-4, Chapter II). Several recent studies have revealed that farmed salmon contains higher levels of POPs including PCBs and PBDEs compared to wild specimens (Easton et al., 2004; Hites et al., 2004, Ohta et al., 2002).

Concentrations of POPs in typical salmon fillets consumed in Singapore are in the range of values reported for farmed salmon.

The PCB congener profile for the majority of the twenty types of seafood analyzed best approximates to the congener profile of Aroclor 1254. A similar match was observed for a variety of marine organisms elsewhere (Miao et al., 2000). The congener BDE-47 (2,2',4,4' tetra-BDE) was proposed as an indicator for PBDE contamination in marine fish (Akutsu et al., 2001). In addition to salmon fillets, BDE-47 has also been detected in selar, seabass and grey prawn samples in this study - seafood which originates from within South-east Asia (MFRD, 1996). Little is known about the occurrence of brominated flame retardants in the environment of Asia (Kemmlein et al., 2003), and the industrial use of technical mixtures of pentabrominated diphenyl ethers in Asia is reported as null (De Wit, 2002). However, the present data suggest that BDE-47, which is the main component of the technical pentabrominated mixture DE-71, is present in the marine environment of South-east Asia and is accumulating in the foodchain.

IX – 4 – 2 Risk assessment

To undertake a risk assessment on the consumption of food, the first step is to compare the levels with the maximum residue limits (MRLs). MRLs for POPs in Singapore (Government of Singapore, 1990) and the United States (USFDA, 2001) are presented in Section II-4-4, Chapter II. Samples were lower than respective MRLs for all POPs.

Data comparison with human consumption studies conducted elsewhere shows that mean daily intake of PCBs from seafood in Singapore represents only 6% of the total daily intake of a whole diet in Italy (Zuccato et al., 1999). The MDI of DDT from seafood in Singapore is 2.5 times higher than for a seafood diet in Italy, according to a study conducted in 1997 (Stefanelli et al., 2004).

The 'oral reference dose' (Oral RfD) is an estimate of the daily exposure of a person to a contaminant that is likely to be without appreciable risk of a deleterious non-carcinogenic effect during a lifetime (USEPA; http://www.epa.gov/iris/). Oral RfD values for POP concentrations in seafood types are presented in Tables IX-2, together with the daily intake of seafood consumed in Singapore. Daily intakes of POPs from seafood are below the oral RfD. The 'cancer benchmark concentration' (Dougherty et al., 2000) represents the exposure concentration at which a lifetime cancer risk equates to one excess cancer death in one million persons. This level is defined as the public health protective concentration in the Congressional House Report to the Food Quality Protection Act of 1996 in the USA. Cancer benchmark concentrations were exceeded for DDTs, heptachlor and PCBs (See Table IX-2). The 'cancer hazard ratio' is the ratio of the MDI for a specific contaminant relative to the cancer benchmark concentration. The cancer hazard ratio represents the extent to which average daily exposure exceeds the benchmark concentration.The cancer hazard ratio of seafood consumption was equal to 1, 1 and 23 for DDTs, heptachlor and PCBs respectively, meaning that, according to Dougherty et al., (2000), a significant number of people are potentially at risk in Singapore over a lifetime of seafood consumption.

However, it worth noting that these calculations are derived from raw tissue analysis and more information is required on the effect of seafood cooking in Singapore on the final load of contaminants ingested. Cooking processes are known to have the ability to decrease the load of POPs in seafood (See Chapter X). It is also important to note that the standard deviation for per capita seafood consumption data in Singapore is large (i.e. 46.3±36.9 and 49.9±40.0 g/day for women and men respectively), where significant levels of variability exist between ethnic subgroups in the population. As an example, a Malay male adult in Singapore consumes 64.9g/day of seafood versus 42.8 g/day for an Indian and 48.2 g/day for a Chinese male adult, respectively (Ministry of Health, Singapore, 2001). The risk associated with seafood consumption is therefore up to 51% higher for a male adult in the Malay community.

IX – 5 Conclusion

This study has proved the ubiquity of POPs in seafood commonly consumed in Singapore. As a result of the use of contaminated fish meals and as discussed in Chapter VIII, levels of POPs in farmed salmon were relatively higher than in any other fish commonly consumed in Singapore. Human health risks associated with the consumption of contaminated seafood exist and maximum exposure criteria are exceeded when considering seafood consumption alone. Further investigation should be undertaken to consider exposure through the whole diet. Chapter X will investigate the effect of cooking on the POPs loading in salmon and evaluate the consequences for risk assessment based on salmon consumption.

CHAPTER X – EFFECT OF COOKING ON THE LOSS OF POPs FROM SALMON

X – 1 Introduction

Recent studies have shown high concentrations of a range of POPs in farmed fish compared with wild specimens, particularly in farmed salmon, seemingly due to the bioaccumulation of POPs from the ingestion of contaminated commercial fish feeds (See Chapter VIII). Farmed salmon was reported to have the highest levels of POPs amongst twenty types of seafood commonly consumed in Singapore (See Chapter IX). Cooking processes, such as baking, frying or boiling are known to reduce the burden of POPs in fish (Zabik and Zabik, 1999; Schecter et al., 1998). However, the mechanisms involved in the transfer and/or degradation of POPs during the cooking process are not clear. As a result, the scientific literature reports inconsistent effects of cooking on the behaviour of POPs in fish (Armbruster et al., 1987; Zabik and Zabik, 1999). Losses of POPs from cooked fish have been investigated in conjunction with cooking temperature and the surface area of fish fillets (Stachiw et al., 1988). One of the major properties of POPs is their lipophilic nature, which is reflected by a high octanol-water partition coefficient (K_{ow}) (Braekvelt et al., 2003). However, no reports exist on the correlation between cooking losses of POPs from fish and tissue lipid changes.

This study was conducted to investigate the loss of POPs, including PCBs, PBDEs, DDT and chlordane congeners, from Atlantic salmon (*Salmo salar*) steaks when subjected to different cooking processes. This is the first report on PBDE loss as a result of cooking of a food

product, where measured POP concentrations before and after cooking were statistically compared with the total loss of lipids from the salmon tissue. A mass balance of POPs has also been calculated to determine the effect of skin removal on the burden of POPs in cooked salmon steaks.

X – 2 Materials and Methods

X – 2 – 1 Sample collection and preparation

Stainless steel tools used for preparation of salmon steaks for cooking were systematically washed with laboratory detergent and rinsed with acetone prior to use. Three Atlantic salmon (*Salmo salar*) specimens, originating from Norway, were purchased chilled and ready gutted from a local market in Singapore. Fish length and weight averaged 75 ± 2 cm and 4.1 ± 0.1 kg respectively. Prior to cooking, the concentration of POPs was measured in the three fish and compared. No significant differences were found between the individual fish. On this basis, one fish was randomly chosen for the study as being representative of a typical salmon consumed in Singapore. Without removal of the skin, steaks, of two cm thickness, were cut with a stainless steel knife and weighed. The steak was divided into the left and right lobes and one side was cooked and the other used as the uncooked reference sample.

X – 2 – 2 Cooking processes

Fish were processed in the Food Science and Technology Department of the National University of Singapore within one day of purchase. Salmon steaks were cooked either by baking, microwave cooking, boiling or pan-frying to attain portions with edible properties comparable to steaks commonly consumed in Singapore. Baking and microwave cooking were performed using an EMO-SRT1 programmable combined electric conventional and microwave oven (Sanyo, Osaka, Japan). Baking was performed for 30 min at 180°C, after preheating the oven for 10 min. Microwave cooking was performed for 5 min using 500 W power. For boiling preparation, steaks were boiled in 1.5 L of water for 5 min. For pan-frying, sunflower oil was used at a temperature of 180°C for 5 min. The oil was sampled before and after frying and analyzed for its POPs content. Internal temperatures of the fish portions were recorded before and after cooking using a 309 Temperature Probe (Corning, NY, USA). The fish skin was immediately removed from the steak after cooking and weighed. Steaks were processed in triplicate for each cooking method, and steaks were randomly selected from along the fish body to minimize the effect of any variation in POPs and lipid content. The triplicates were also analysed to compare left and right steak lobe concentrations, to ensure that there was no significant variation in the transverse symmetry of POP concentrations.

IX – 2 – 3 POPs analysis

Muscle samples were homogenized in a stainless steel blender and skin tissue was cut into fine pieces using a scalpel. Sample size for analysis was 4 g for muscle tissue, 2 g for skin and 1 g for the cooking oil used for pan-frying. The use of MAE extraction and the POPs

presentation have been presented and validated in Chapter IV. After MAE, an aliquot (1.5 mL) was reserved for extractable lipid determination using a gravimetric method (See Section IV-4-9, Chapter IV). In the present study, PBDEs refers to the sum of BDE-47, 99 and 100. The details on the congeners for chlordanes, DDTs and PCBs are presented in Section III-6, Chapter III. PCB congeners 55 and 61 were used as recovery standards in all samples. The standard reference material SRM 2978 - green mussel tissue - and SRM 1588a – cod liver oil - were analyzed to validate the complete analytical method.

X – 3 Results

IX – 3 – 1 Quality assurance

Recoveries of surrogate standards PCB 55 and 61 were satisfactory with an average recovery of 98% ± 15%. The certified values for PCBs, DDTs and chlordanes, were achieved with an average recovery of 89% ± 19% for SRM 2978 and 93% ± 19% for SRM 1588a, with typically less than 30% difference between analytical and certified values, except for trichloro-PCBs.

V – 3 – 2 Analysis of raw muscle and skin samples

The lipid content of the salmon steaks ranged between 8.3 to18.3% and 18.4 to 44.0% in the muscle and skin tissues respectively (Figure X-1). As the values are >5%, the lipid content determination can be validated (See Section IV-4-9, Chapter IV). Median lipid contents were significantly higher in the muscle and skin tissues of the twelve salmon steaks closer to the fishes head compared with the six closer to the tail (Mann-Whitney, $p<0.05$).

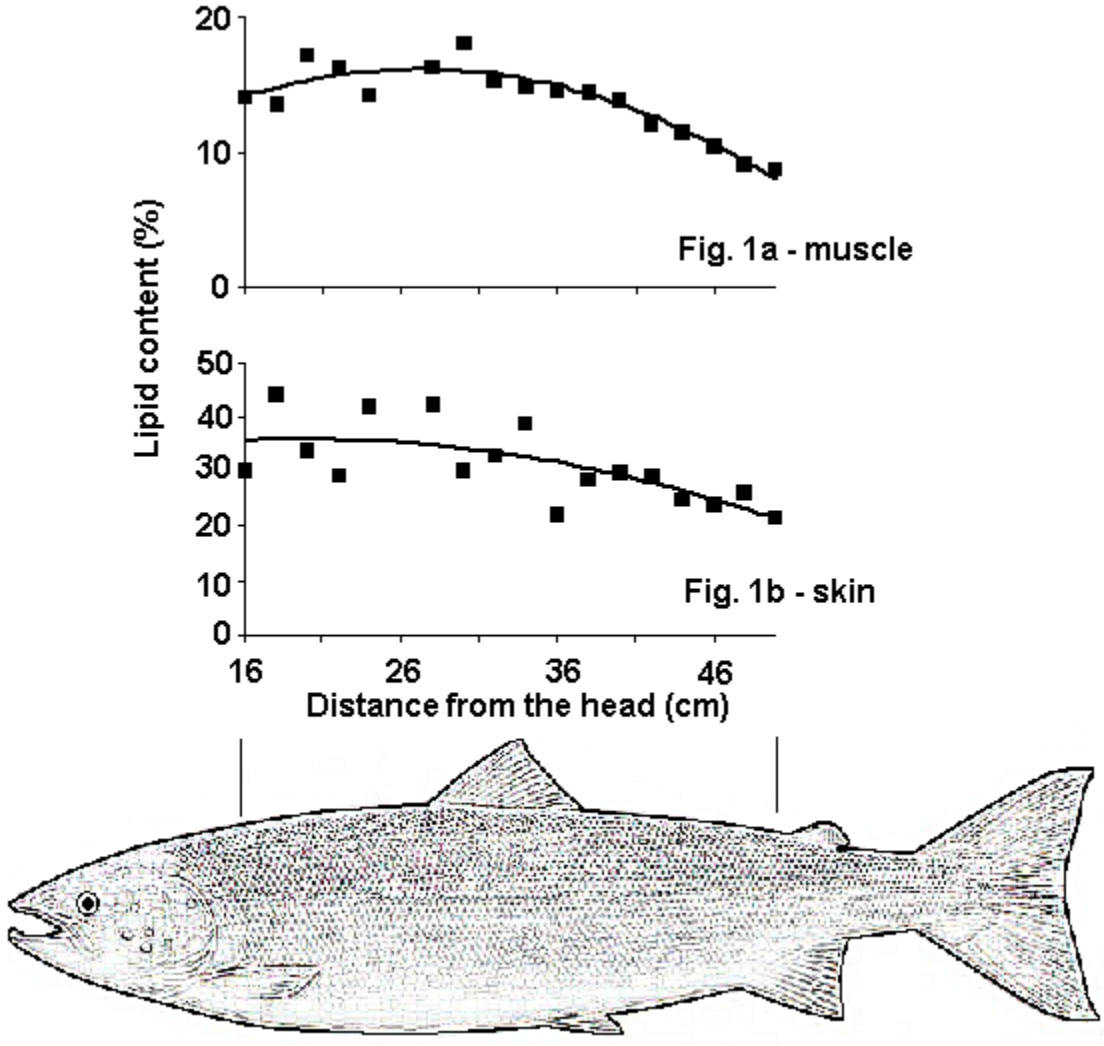

Figure X-1: Lipid contents in salmon muscle (1a) and skin (1b) in steaks taken from different positions of the fish body.

Limits of detection for POPs ranged from 0.01 ng/g to 3.3 ng/g ww in muscle tissue and from 0.02 ng/g to 2.5 ng/g ww in skin, depending on the contaminant. Levels of POPs between the left and right lobes of three steaks were compared, at different positions on the fish body. The average RSD ranged between 6-13% for muscle tissue and 8-32% for skin on a wet weight basis. Average RSD decreased to 5-9% for muscle tissues and 9-16% for skin on a lipid

weight basis. Therefore, transverse symmetry of POPs in the salmon steaks meant that raw and cooked tissue could be compared independently of the actual steak lobe cooked.

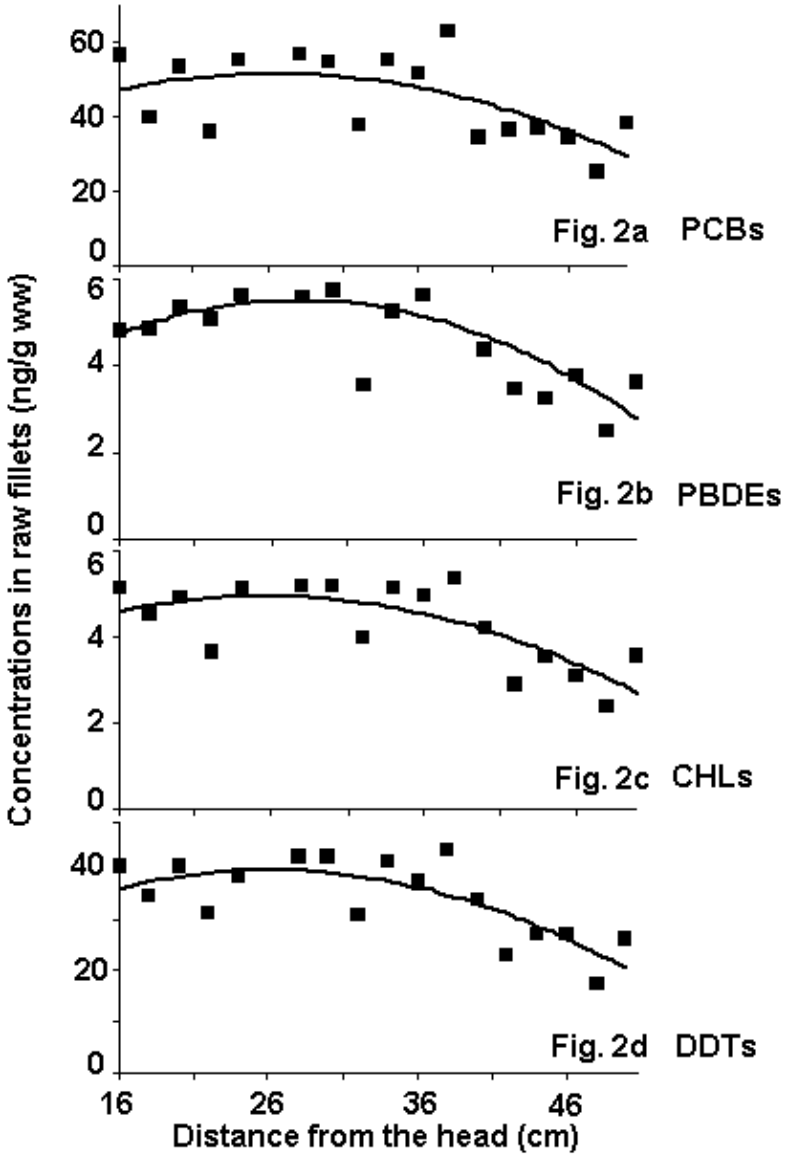

Figure X-2: Concentrations of PCBs (2a), PBDEs (2b), chlordanes (2c) and DDTs (2d) in raw salmon fillets taken from different positions of the fish body (ng/g wet weight).

Concentrations of PCBs, PBDEs, chlordanes and DDTs were measured in the raw salmon steaks taken from various positions along the fish body (See Figure X-2). Total levels in the

raw muscle tissue ranged between 25.1-62.9 ng/g ww for PCBs, 2.5-7.6 ng/g ww for PBDEs, 2.4-5.3 ng/g ww for chlordanes and 17.5-43.8 ng/g ww for DDT. Total levels in the raw skin ranged between 43.8-132.5 ng/g ww for PCBs, 4.1-11.5 ng/g ww for PBDEs, 5.6-15.0 ng/g ww for chlordanes and 37.7-97.8 ng/g ww for DDT. BDE-47 was the dominant PBDE congener, representing generally more than 70% of the total PBDE concentration.

In raw salmon steaks, skin represented 6.5±1.5% of the total weight and contained an average of 13±4% of the total load of POPs. Median concentrations on a wet weight basis for all POPs were significantly higher in the salmon muscle and skin tissues of the twelve steaks closer to the fishes head compared with the six closer to the tail (Mann-Whitney, $p<0.005$). Since POPs are lipophilic, tissue POPs concentrations and lipid contents can be expected to be positively correlated. As a consequence, there was no significant difference between POPs concentrations on a lipid weight basis (lw) between the steaks, irrespective of body location (Mann-Whitney, $p<0.05$).

IX – 3 – 3 Effect of cooking

The total burden of POPs in the salmon steaks when subjected to each cooking process was derived by multiplying the weights of tissues (muscle or skin) by their respective analyte concentrations, before and after cooking. Losses of POPs, expressed as a percentage of the pre-cooked concentration, were significantly higher for skin than muscle tissues, with an average loss of 37% versus 23% (Mann-Whitney, $p<0.005$). As a result, skin represented an average of 11±3% of the total load of POPs in the cooked steaks for the various cooking processes, i.e. slightly less than for the raw steaks.

Losses of POPs as a result of the various cooking processes are presented in Table X-1, with and without skin removed. Overall, the average loss of POPs equated to 35%±13%, ranging from 13 to 51% depending on the contaminant and the cooking method used. Cooking resulted in an average loss of 26±15% relative to the initial POP load in the raw salmon steak. The removal of the skin from the cooked salmon steak resulted in a further average loss of 9±3%. The loss of POPs did not differ significantly between cooking methods (Kruskal-Wallis, $p<0.05$). Final internal temperature in the steaks during cooking averaged 80±12°C, and there was no significant correlation ($p<0.05$) between the loss of POPs and cooking temperature.

	Pan-frying		**Microwave cooking**		**Boiling**		**Baking**	
	skin on	**skin off**	**skin on**	**skin off**	**skin on**	**skin off**	**skin on**	**skin off**
PCBs	36±11	44±11	23±14	30±12	28±16	38±16	28±13	36±13
DDTs	31±16	41±14	21±15	29±12	25±5	37±6	19±21	28±19
CHLs	23±13	34±12	18±16	28±14	23±16	35±15	13±24	23±22
PBDEs	42±15	48±14	25±24	31±22	32±11	40±11	44±20	51±27

Table X-1: Loss (%) of POPs from Atlantic salmon steaks (with and without skin) for different cooking methods relative to total initial loading in raw steak.

The percentage loss of lipids, plotted as a function of the initial lipid content, is shown in Figure X-3. A higher initial lipid content in a particular steak (muscle tissue and skin) led to a higher relative loss of lipids. Thus, leaner steaks, close to the tail of the fish, with lipid

contents of less than 14%, showed a loss of less than 20% of the initial lipid burden after cooking. On the contrary, steaks closer to the belly and the head, with lipid contents in excess of 15%, typically showed losses of greater than 20% of the initial lipid burden. It should be noted that, in Figure X-3, the error bar on the trend becomes larger for lipid content higher than 15%.

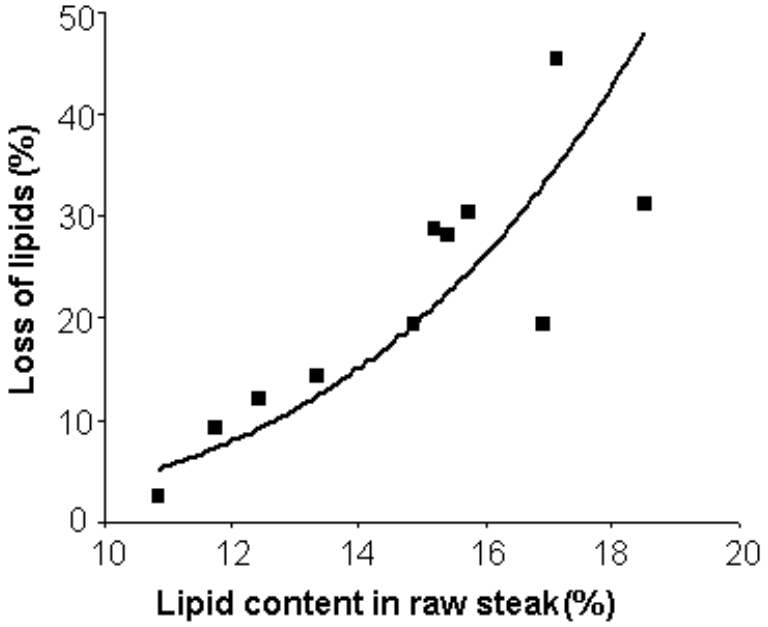

Figure X-3: Percentage loss of lipids from salmon steaks after cooking relative to initial lipid content.

Losses of POPs relative to lipid loss in tissues are shown in Figure X-4. Cooking losses for all POPs analysed were linearly and positively correlated with the loss of lipids, with a ratio of 1:1. The ratios for PBDEs (1.44:1) were higher than for PCBs (1.22:1), DDTs (1.08:1) and chlordanes (0.93:1). As a result, cooking raw steaks with higher lipid content resulted in a proportionally higher loss of contaminants.

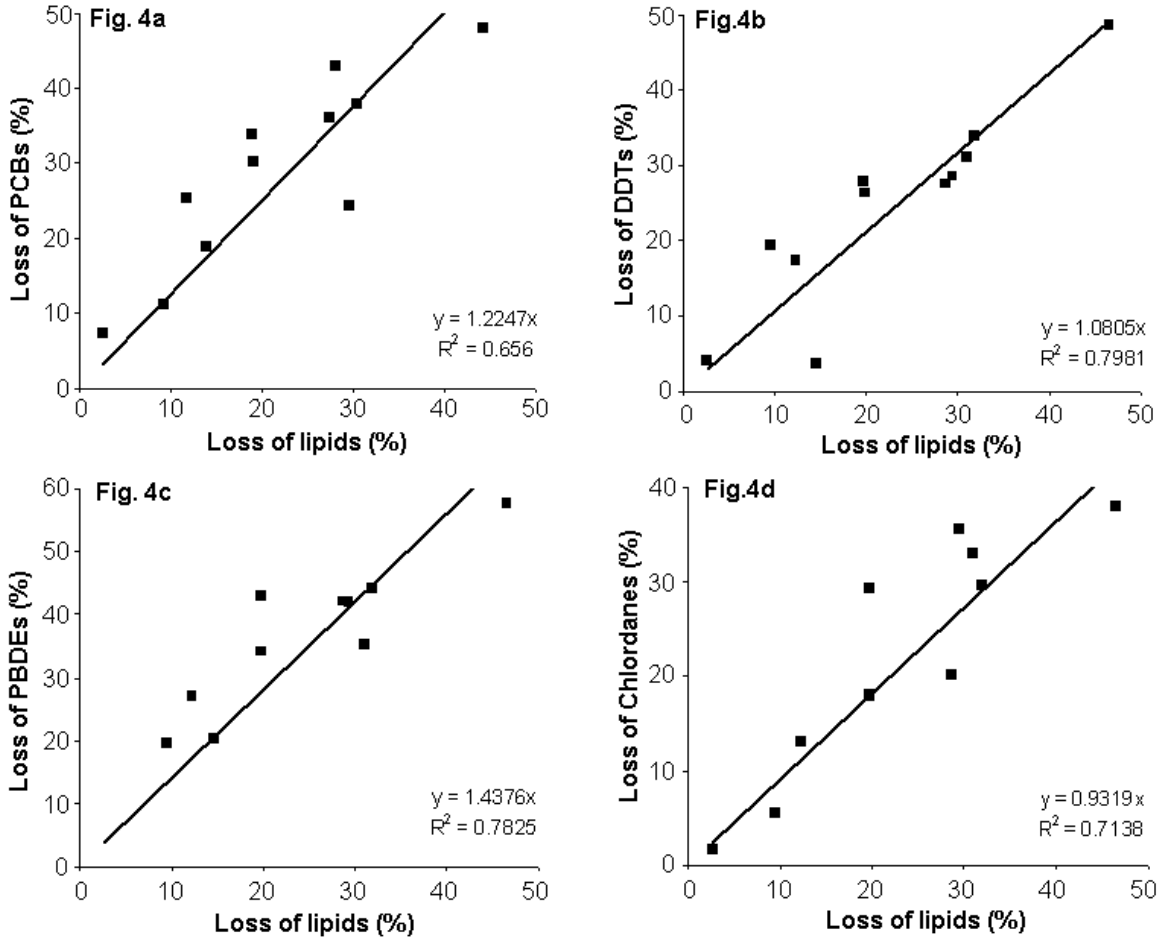

Figure X-4: Percentage loss of lipids from salmon steaks after cooking relative to initial lipid content.

The average losses for PCBs and PBDE congeners, according to degree of congener chlorination and bromination, is presented in Figure X-5. In general, higher levels of chlorination or bromination resulted in a higher loss, although differences were not significant.

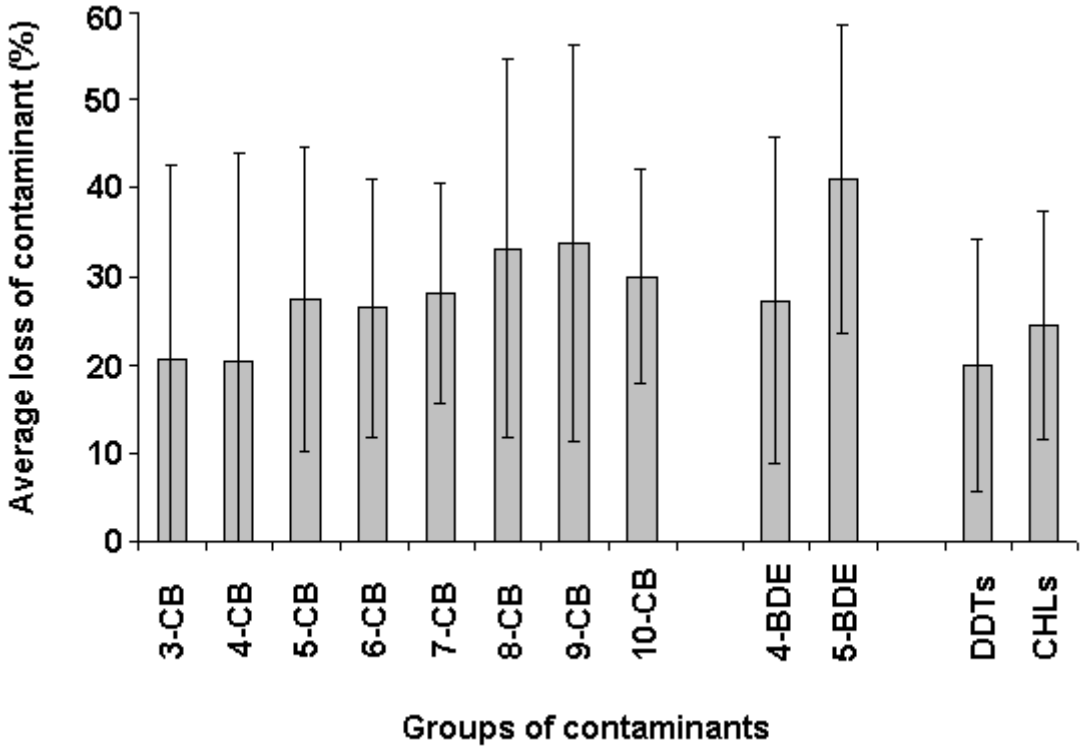

Figure X-5: Average contaminant loss in salmon steaks (muscles and skin) for specific groups of contaminants.

Figure X-6 shows the total load of POPs in raw and cooked salmon steaks for the different cooking methods. As a result of the relative differences in lipid losses, the initial significant differences in the burden of POPs in raw steaks taken from different parts of the fish body were no longer significant after cooking (Mann-Whitney, $p<0.05$). Therefore, the relative levels of contaminants in cooked salmon steaks were independent of the initial burden in the raw salmon steaks.

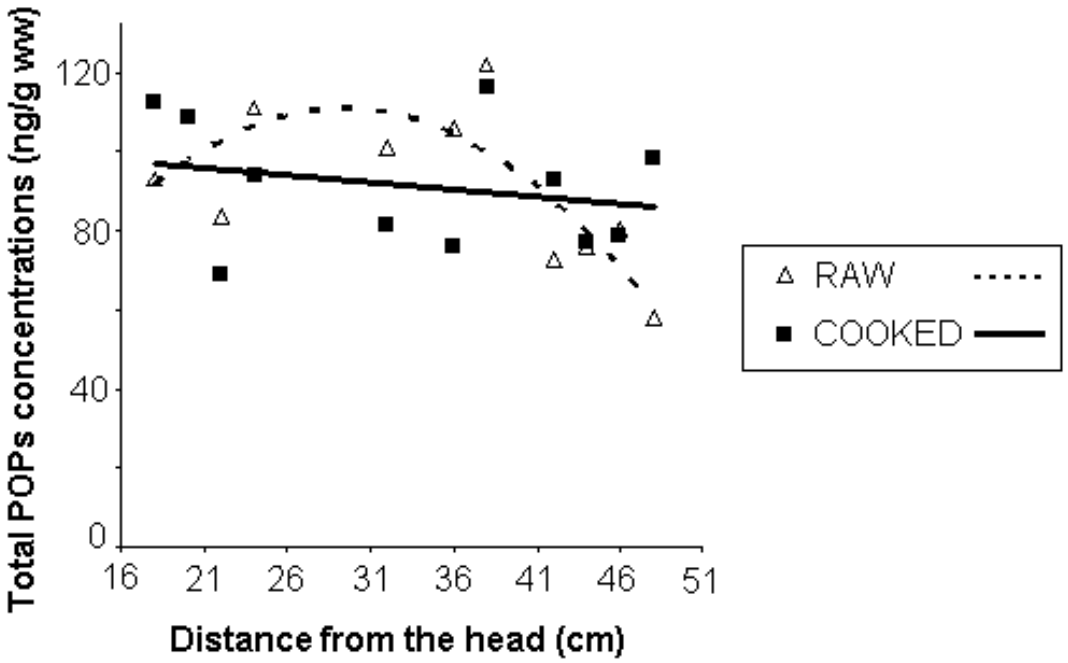

Figure X-6: Total concentration of POPs in raw and cooked salmon steaks.

The cooking oil used for pan-frying salmon steaks was analyzed before and after use to determine if POPs lost from the salmon steak during cooking could be accounted for. Concentrations of contaminants in the oil increased from 0.9 to 118.0 ng/g ww for PCBs, <0.3 to 10.3 ng/g ww for PBDEs, 0.1 to 11.7 ng/g ww for chlordanes and <2.3 to 92.9 ng/g ww for DDTs. A mass balance calculation reveals that the enrichment of POPs in the cooking oil used for frying averaged 117%±60% of the amount lost from the cooked salmon steaks.

IX – 4 Discussion

IX – 4 – 1 Concentration of POPs in raw salmon steaks

Several recent studies have revealed that farmed salmon contains higher levels of POPs including PCBs and PBDEs relative to levels in wild specimens (Easton et al., 2004; Hites et al., 2004, Jacobs et al., 2002; Ohta et al., 2002). The concentrations of POPs in the salmon steaks used in this study were in the range of values reported for farmed salmon. It is worth noting that the salmon specimen used in the study had a commercial weight of 4 kg, where Makarewicz et al. (2003) recorded POPs concentrations 2 to 3 times higher in a salmon specimen weighing 12-14 kg than in a 4 kg specimen. The congener profile for PCBs was dominated by penta- and hexa-chlorinated biphenyls, which is typical of the congener profile found in salmon (Jackson et al., 2001). BDE-47 is considered as the most abundant PBDE congener in wildlife (Marsh et al., 2004), including salmon (Jacobs et al., 2002). In this study, BDE-47 represented more than 70% of the total PBDE burden in both salmon muscle and skin.

Fish skin generally contains higher levels of POPs than muscle tissues (Zabik et al., 1996). In this study, levels of POPs in raw salmon skin represented only 13±4% of the total load of POPs in the salmon steak, which is lower than levels reported for other fish species, such as white croakers, 33-40% (Davis et al., 2002). The lipid concentration range in the salmon muscle tissue used in this study (i.e. 8.3-18.3%) is similar to levels reported previously (Jacobs et al., 2002). Lipid levels in the raw muscle tissue from the tail of the salmon were

approximately half those in muscle tissues from near the belly of the fish, and this can be attributed to the higher fat content in the belly flap (Zabik and Zabik, 1999).

V – 4 – 2 Effect of cooking on levels of POPs

The loss of lipids was the controlling factor in the loss of POPs from the salmon steak, where variation in the loss of POPs was independent of the cooking method used. The ratio of the total loss of POPs to the loss of lipids was close to 1:1 (See Figure X-4), indicating that POPs transferred out of the fish tissue together with fish lipids during cooking. In the case of pan-frying, the amount of POPs lost by the fish approximated to that retrieved in the cooking oil, suggesting that melted fish lipids transferred POPs into the cooking oil. Previous studies have reported that increased cooking temperature or surface area of fillets results in a higher loss of lipids (Stachiw et al., 1988). This was likely to have resulted from an increase of lipid loss when the cooking temperature or the surface area increases, although this was not measured in this study. Advisory agencies in the US indicate that smoke curing of fish results in a significantly greater loss of POPs compared with other cooking processes, although it also increases the burden of polynuclear aromatic hydrocarbons (USEPA, 2000). The study used as reference for this statement, i.e. Zabik et al. (1996) noted that "visual observation during smoking indicated much of the additional loss was fat". Therefore, the efficiency of POPs loss from smoking might be also attributed to a greater loss of lipids. Overall, it would appear that the reduction of POP burden in cooked fish is a result of efficient lipid removal during the cooking process rather than due to the type of cooking method used.

Of all the cooking processes used in this study, PBDE losses were relatively greater than for other POPs. There are no comparative data available in the literature on the loss of PBDEs in food as a result of cooking. Losses were greater for congeners with higher levels of chlorination for PCBs, and bromination for PBDEs. Zabik and Zabik (1999) noted that losses of PCBs with lesser chlorination levels (3-CB, 4-CB) were lower than for higher levels of chlorination (5-, 6- and 7-CBs). Physical properties of PBDE congeners differ in terms of inter-media partitioning, where PBDES have a higher octanol–air partition coefficient (K_{oa}) and octanol–water partition coefficient (K_{ow}) than DDT or PCB congeners with a similar level of substitution (Albaiges, 2003; Braekvelt et al., 2003).

Overall, the initial burden of POPs in the salmon steaks decreased by 26±15% after cooking with an additional loss of 9±3% when the skin was removed from the cooked steak. The mean loss values for the Atlantic salmon are in the range of those reported for cooked lake trout or Pacific salmon (USEPA, 2000). Human health effects associated with the consumption of cooked salmon can be considered to decrease proportionally, and it can be concluded that cooking has a beneficial reduction on the burden of POPs in salmon steaks. In the previous chapter, mean daily intake of PCBs and DDTs from seafood for the general Singaporean population was lower than the oral reference dose (non-carcinogenic effects), but exceeded the cancer benchmark concentration set by the USEPA. Amongst twenty types of raw seafood analyzed, salmon fillets represented the major source of PCBs and PBDEs for seafood consumption. Based on the present results, an average reduction of the POPs load by 26% following cooking would decrease the mean daily intake of POPs proportionally. The consequence is that the mean daily intake of DDT would then be below the cancer

benchmark concentration; however the cancer hazard ratio for PCBs (17) would be still significantly greater than one - even when taking into account the cooking of the seafood. Based on the present results, adjustments taking cooking effects into account are also necessary in other risk assessment studies. For example, Hites et al. (2004) reported levels of POPs in raw salmon fillets from both wild and farmed specimens, and used USEPA methodology to assess the human health risk associated with salmon consumption. Farmed Atlantic salmon was found to have high levels of POPs with adverse health risks after human consumption. However, the lipid content of salmon varies between 3.4 and 20.0 % (fresh weight) with significant variation according to fish size, age and species type (Easton et al., 2002; Makarevisz et al., 2003; Jacobs et al., 2002). Farmed salmon has a higher lipid content than wild specimens (Makarevisz et al., 2003), but will have the greatest loss of lipids after cooking. The decrease in the POP load after cooking is significant enough to affect the risk assessment calculations, meaning that the difference in risk between consumption of farmed and leaner wild specimens may not be as important as suggested by Hites et al. (2004).

IX – 5 Conclusion

Cooking effects and skin removal dramatically decrease the amount of POPs in fish portions destined for human consumption. Removal of lipids during cooking is the critical factor in reducing POP exposure from consumption of cooked salmon. This parameter is crucial in risk assessment calculations and should not be omitted. This is all the more essential since the toxicological assessment of food products can have a large impact on the economical success of the food industry involved, particularly for fresh aquaculture products such as salmon.

XI – CONCLUSIONS & SUGGESTIONS FOR FURTHER STUDIES

XI – 1 Summary of main conclusions

In conclusion, this work has fulfilled its objectives and provides new insights on the analysis of POPs, their occurrence in the marine biota and their potential risks for human beings and the environment. These studies can serve as a base for further work on our understanding of POPs in the South-east Asian region. The main conclusions obtained from this research are summarized as:

Chapter IV. Microwave assisted chemistry applied to the determination of POPs in marine biological samples.

Refer to Section I-2, Chapter I for research objectives. A fast and robust analytical method, using MAE, was optimized for the analysis of POPs, including PBDEs, in marine biota tissues. The sensitivity of the whole method was comparable with other studies and made possible the analysis of POPs, including PBDEs, at very low level in the marine biological tissues. Several levels of quality assurance were adopted for the whole analytical procedures and results were satisfactory. The choice of MAE, instead of conventional extraction techniques such as Soxhlet, resulted in important savings in terms of solvent consumption and extraction time and enabled the analysis of a large number of samples for this project.

Chapter V. The green mussel, *Perna viridis*, as a bioindicator in Singapore.

Refer to Section I-2, Chapter I for research objectives. POPs were detected in Singapore's marine environment and levels reflect the ubiquity of such pollutants in local marine biota. The green mussel, *Perna viridis*, was used successfully as a bioindicator species for Singapore's marine environment for a range of contaminants including PCBs, organochlorine pesticides and, for the first time, PBDEs.

(a) POPs, such as PCBs, DDTs, chlordanes, heptachlor and mirex were detected in Singapore's marine environment in 2002 and 2004. PCNB and HCB were not detected. Penta-BDEs were discovered in Singapore' coastal waters, which represents their first recorded occurrence in South-east Asia.

(b) Background levels of POPs in *P. viridis* correspond to the lower range of values reported in *P. viridis* tissues from elsewhere in Asia and America. Peak levels of PCBs and organochlorine pesticides are in the range of values for green mussel tissues from the marine environment of Hong Kong, which is regarded as heavily contaminated. PBDE levels in mussel tissues from Singapore are up to an order of magnitude greater than available data from elsewhere.

(c) The geographical distribution of POPs in green mussel samples revealed the presence of "hot spots" of contamination associated with industrial and shipping activities in Singapore.

(d) Levels of POPs in mussel samples were correlated with the androgenic hormone activity of mussel extracts using a human-cell based bioassay. The combination of the use of green mussels and the human-cell based bioassay also offers a new

understanding of the presence and potential impacts of endocrine disrupting chemicals on marine biota.

Chapter VI. POPs in mangrove food webs of Singapore.

Refer to Section I-2, Chapter I for research objectives. POPs were quantified in twenty-four mangrove species collected at two sites in Singapore. This is the first such data reported for mangrove ecosystems in Singapore and results confirm the ubiquity of POPs, including PBDEs, in the marine environment.

(a) A biomagnification phenomenon was observed amongst the species collected and analysed from both mangrove sites studied. Thunder crabs and fish displayed the highest POP levels.

(b) Congener profiles of PBDEs varied amongst mangrove biota species suggest different metabolic pathways of the flame retardants. Similarly, crab species showed an ability to metabolize chlordane.

(c) The two mangrove sites, on both sides of the Straits of Johore, do not show any clear differences in terms of contaminant load. However, mangrove organisms collected in S. Khatib Bongsu generally have higher levels of PCBs and PBDEs.

(d) Comparisons with other studies suggest potential ecotoxicological impacts for organisms at higher trophic levels in the mangrove food web, including mammals and birds.

Chapter VII. Exposure of aquacultured oysters to pollutants in Singapore's coastal waters.

Refer to Section I-2, Chapter I for research objectives. The comparative growth rates and POPs bioaccumulation were successfully monitored in Pacific oysters, *Crassostrea gigas*, at two sites in Singapore, one 'clean' and one 'contaminated'.

(a) The accumulation of POPs, including PCBs, DDTs, chlordane and PBDEs, was observed in oyster tissues cultured in Singapore's waters. The biological half-life of POPs in *C. gigas* was less than 33 days.

(b) The growth of oysters and their POPs burden were significantly different at the two sites, revealing that pollution can have potentially adverse effects for the oyster aquaculture industry in Singapore. In particular, POPs and TBT represents a specific threat to both the yield and quality of oyster tissues.

(c) The effects of pollution on oysters were found to be reversible, where transplantation to a 'clean' site will allow the organism to recover in terms of growth rate and tissue quality.

Chapter VIII. Transfer of DDT in aquaculture seabass.

Refer to Section I-2, Chapter I for research objectives. The ingestion exposure of Asian seabass to *p,p*'-DDT pesticide were evaluated in a simulated aquaculture system. Such results underline the importance of fish meal quality on the tropical aquaculture product, and therefore on human food safety.

(a) The partitioning of *p,p*'-DDT congeners in the fish was a function of tissue lipid content. In particular, 14±7% of the DDT ingested by the seabass is partitioned to the edible fish fillet.

(b) Virtually all *p,p'*-DDT in the fish meal was bioaccumulated in the fish, with an uptake efficiency of 98%. The metabolisation of *p,p'*-DDT, which occurs mainly in the liver, resulted in the degradation of 2.5% of *p,p'*-DDT into *p,p'*-DDD. Results show that bioaccumulation mechanisms following ingestion exposure to *p,p'*-DDT at environmentally relevant levels (ng/g range in fish meal) are different from the unrealistic dosage levels used in previous environmental modeling studies.

(c) The continual introduction of POPs, such as *p,p'*-DDT, into aquaculture systems via contaminated fish meals is likely to result in a substantial increase of the pollutants in the fish portions destined for human consumption.

Chapter IX. POPs in typical seafoods consumed in Singapore.

Refer to Section I-2, Chapter I for research objectives. The levels of several POPs were measured in the edible portions of 20 different seafood types consumed in Singapore. This is the first report of a contaminant risk assessment based on seafood consumption in South-east Asia.

(a) POPs were measured in the edible portions of twenty different seafood types commonly consumed in Singapore. Highest levels of POPs were measured in farmed fish fillets and green mussels. Penta-BDEs were also detected in seafood

imported from neighboring countries (e.g. selar fish), underlining the ubiquity of POPs in South-east Asia.

(b) The mean daily intake of DDTs, PCBs and PBDEs from seafood reached 3.0, 3.0 and 0.1 ng/kg body weight per day respectively for the general population of Singapore.

(c) Levels of POPs in seafood were below maximum residue limits set in Singapore and in the United States.

(d) The mean daily intake of contaminants from seafood was calculated for the general population of Singapore. Daily intakes of POPs from seafood are below the oral reference dose set by the United States Food and Drug Administration. Daily intake of DDTs, heptachlor and PCBs in seafood exceeded the conservative cancer benchmark concentrations set by the US EPA, suggesting that a significant number of people are potentially at risk in Singapore over a lifetime from seafood consumption.

Chapter X. Effect of cooking on the loss of POPs from salmon.

Refer to Section I-2, Chapter I for research objectives. Cooking effects and skin removal dramatically decreased the amount of POPs in fish portions destined for human consumption.

(a) Cooking resulted in an average loss of 26±15% relative to the initial POP load in the raw salmon steak. The loss of POPs did not differ significantly between cooking methods.

(b) The removal of the skin from the cooked salmon steak resulted in a further average loss of 9±3%.

(c) Removal of lipids during cooking is the critical factor in reducing POP exposure from consumption of cooked salmon.

(d) Cooking effects are a crucial parameter in risk assessment calculations and should not be omitted. The mean daily intake of DDT and heptachlor in Singapore would then be below the cancer benchmark concentration; however the cancer hazard ratio for PCBs would be still significantly greater than a critical value of one - even when taking into account the cooking of the seafood.

XI – 2 Suggestions for further studies

Specific recommendations for future work include:

8. To determine the routes of PBDEs to the environment, especially since the demand for the commercial penta-BDE formulation was reported to be null in Asia (De Wit, 2002). Singapore, and more generally Asia, therefore may represent a significant source of PBDEs at a global scale.
9. To monitor, on a regular scale, the levels of contaminants in green mussels so as to obtain a long term temporal trends of POPs pollution in Singapore's marine environment.
10. To study the ecotoxicological impact of POPs on marine organisms, birds and mammals. A combination of analytical chemistry and biochemistry / immunochemistry to measure POPs and biomarkers of POP exposure would appear as an effective combination of analytical tools for this purpose.

11. To further investigate human exposure to POPs, and associated health risks, in the region. Eventually, the occurrence of POPs in human tissues should be investigated so as to obtain a clear picture on the toxicological risks for Singapore's population.

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Appendix A: GC-MS parameters using EI-SIM mode

Analyte	Retention time (min) [a]	Quantification ion	Confirmatory ion
γ-chlordane	21.6	373	375
α- chlordane	22.5	373	375
heptachlor	14.7	272	274
heptachlor epoxide	20.0	353	355
PCNB	9.3	235	237
p,p'-DDT	29.6	235	237
p,p'-DDD	27.2	235	237
p,p'-DDE	24.4	246	248
endosulfan I & II	22.5, 26.4	339	341
endosulfan sulfate	29.0	272	274
mirex	35.7	272	274
HCHs	7.8 to 11.5	217	219
3-CBs	10.3 to 14.4	256	258
4-CBs	16.3 to 21.2	290	292
5-CBs	20.6 to 28.1	324	326
6-CBs	25.4 to 35.2	360	362
7-CBs	30.6 to 35.5	396	398
8-CBs	36.0 to 39.2	426	428
9-CBs	37.9 to 40.5	462	464
10-CB	41.5	496	498
BDE-47 [b]	34.2	326	484,486
BDE-99 [b]	40.2	404	406, 565,567
BDE-100 [b]	39.0	404	406, 565,567
BDE-153	43.5	641	643
BDE-154	42.4	641	643

[a] Example of retention times obtained using GC program 3 as described in Section III-6, Chapter III. [b] The choice of the ions monitored is dependant on the presence of interferences on the chromatogram (ElJarrat et al., 2004).

Appendix B: Details on the mussel batches collected in Pulau Ubin between July 2003 and January 2004.

Sampling date	Size range (mm)	Size (mm)	n	Sex ratio [a]	Moisture content (%)
16 July 2003	10-29	22±4	40	n.a.	82±4
	30-49	41±6	20	1.2	80±3
	50-69	59±6	19	1.1	87±2
	70-79	74±3	20	4.0	84±1
	80-99	85±5	16	1.0	79±2
	>100	113±10	11	0.8	85±1
3 September 2003	10-29	24±4	40	1.3	86±4
	30-49	38±6	20	1.5	80±4
	50-69	57±5	19	1.1	87±0
	70-79	72±2	10	1.0	82±10
	80-89	85±4	8	3.0	86±2
	90-99	94±2	13	0.4	81±3
	>100	106±7	5	1.5	82±2
8 October 2003	30-49	45±4	20	1.0	87±3
	50-69	57±6	20	1.0	88±0
	70-79	74±3	11	1.2	88±0
	80-89	83±3	3	2.0	87±2
	90-99	93±3	6	0.2	87±1
	>100	104±2	2	1.0	89±4
12 November	30-49	47±3	20	0.8	88±2
	50-59	56±2	20	0.8	84±2
	60-69	64±2	20	1.0	82±4
	70-79	74±3	14	0.8	79±3
	80-99	85±0	2	0.0	85±2
	>100	107±4	7	0.2	84±2
11 December	10-29	19±2	26	n.a.	84±2
	30-49	39±4	3	2.0	88±2
	50-59	55±3	20	0.8	86±1
	60-69	64±3	20	1.9	82±3
	70-79	74±4	16	0.2	81±0
14 January 2004	10-29	20±4	39	0.8	81±0
	30-49	34±3	20	0.5	85±0
	50-69	63±5	20	1.9	81±3
	70-79	74±3	22	0.8	82±3
	80-99	84±3	7	6.0	83±1
Total			**579**	**1.0[b]**	

[a] Sex ratio refers to the ratio of males to females. [b] Average for all the mussels.

Appendix C: Details on the mangrove biota samples collected in April 2004.

Common name	Site[a]	n	Size (mm)	Weight (g)	Organs selected for analysis	Moisture content of sample (%)	Lipid content of sample (%)
Algaes							
Green algae	W	n.a.	n.a.	50	whole	87.9 ± 0.1	BLD
	E	n.a.	n.a.	50		80.7 ± 4.2	0.2 ± 0.3
Red algae	W	n.a.	n.a.	50	whole	88.4 ± 1.4	0.2 ± 0.3
Polychaetes							
Nereid worm	W	≈ 50	n.a.		whole	93.9 ± 2.4	1.3 ± 0.3
	E	≈ 50	n.a.			89.0 ± 0.2	0.8 ± 0.0
Tube worm	W	25	n.a.	2.8	worm (tube removed)	84.5 ± 3.5	0.5 ± 0.2
	E	25	n.a.	3.2		83.6 ± 0.3	1.6 ± 0.6
Molluscs							
Nerite snail	W	30	27 ± 3	5.2 (1.6) [b]	whole soft tissues	79.8 ± 2.8	1.3 ± 0.1
	E	25	17 ± 2	2.5 (0.6) [b]		81.6	0.6 ± 0.3
Drill shell	W	27	41 ± 3	8 (2) [b]	whole soft tissues	63.3 ± 8.7	1.7 ± 0.1
	E	40	33 ± 3	4 (1) [b]		76.4 ± 4.9	0.8 ± 0.2
Rodong shell	W	29	76 ± 5	37 (6) [b]	whole soft tissues	77.6 ± 0.9	1.2 ± 0.2
	E	30	81 ± 10	95 (13) [b]		75.1 ± 2.1	1.4 ± 0.2

Green mussel	W	25	78 ± 8	10 (5) [b]	whole soft tissues	85.3 ± 1.6	1.0 ± 0.2
Lokan clam	W	7	84 ± 7	210 (43) [b]	whole soft tissues	84.2 ± 0.1	1.8 ± 0.0
	E	22	75 ± 5	134 (31) [b]		86.8 ± 0.1	0.9 ± 0.3
Leaf oyster	W	25	57 ± 6	n.a.	whole soft tissues	78.0 ± 0.0	2.1 ± 0.3
Mangrove oyster	E	6	161 ± 11	534 (53).	whole soft tissues	80.2 ± 0.8	1.5 ± 0.1
Crustaceans							
Barnacles	W	170	shell	0.03	whole soft tissues	79.4 ± 0.8	1.2 ± 0.7
	E	160	2 to 5	0.03		75.7 ± 0.2	1.2 ± 0.6
Snapping prawn	W	20	31 ± 3 [c]	0.6 ± 0.2	meat	74.7± 1.4	2.0 ± 0.7
	E	16	32 ± 13 [c]	1.0 ± 1.6		80.8± 0.5	1.1 ± 0.4
Marine prawn	E	10	120 ± 16 [c]	9 ± 3	meat	77.4 ± 1.2	0.9 ± 0.3
Tree climbing crab	W	5	42/36 [d]	36 ± 20	whole head content	82.2 ± 0.1	0.9 ± 0.2
	E	6	23/20 [d]	6 ± 3		79.9 ± 1.8	0.8 ± 0.5
	W				eggs		3.1
	E						3.1
Thunder crab	W	5	41/30 [d]	18 ± 8	whole head content	84.1 ± 5.4	2.1 ± 0.5
	E	7	35/23 [d]	11 ± 8		75.0 ± 1.3	2.1 ± 0.5
Fish							
Half-beak	W	5	175 ± 19	16 ± 6	fillet	80.8 ± 0.2	0.3 ± 0.0
	E	5	136 ± 9	8 ± 2	fillet	80.5 ± 0.8	1.2 ± 0.1

Giant mudskipper	W	3	140 ± 12	36 ± 12	fillet	81.6 ± 0.3	0.4 ± 0.0
					eggs		7.5
Green spotted goby	E	4	88 ± 11	11 ± 2	fillet	78.9 ± 1.2	1.0 ± 0.2
	E				liver		16.5
	E				eggs		18.3
Glass perchlet	W	20	78 ± 4	4.8 ± 0.8	fillet	75.8 ± 1.6	1.0 ± 0.1
Mangrove cardinalfish	E	3	114 ± 12	30 ± 13	fillet	76.9 ± 13.0	0.6 ± 0.4
	E				liver		16.4
Mullet	W	2	179 ± 8	60 ± 7	fillet	77.8 ± 1.5	3.4 ± 0.2
	W				liver		10.9
	E	6	95 ± 9	13 ± 4	fillet	77.9 ± 0.7	3.2 ± 0.2
Archer fish	W	3	183 ± 3	117 ± 12	fillet	76.7 ± 2.5	1.4 ± 0.0
	W				liver		7.1
	E	1	83	11	fillet	79.9	1.1
Green chromide	W	3	252 ± 14	350 ± 52	fillet	79.7 ± 0.5	0.3 ± 0.0
	W				liver	68.9 ± 3.9	5.2
	E	3	138 ± 51	72 ± 50	fillet	79.9 ± 2.8	1.8 ± 1.3
	E				liver		17.9

[a] W: Sungei Buloh, E: Sungei Khatib Bongsu. [b] Typical weight of the whole organism; in brackets is the weight of soft tissues. [c] Body length without antennae.[d] Carapace width/length.

Appendix D: POPs concentrations (ng/g ww) in the mangrove biota samples collected in April 2004.

Common name	Site[a]	CHLs	DDTs	PCBs	PBDEs	HCHs	Endosulfans
Green algae	W	0.37 ± 0.12	0.18 ± 0.08	1.1 ± 0.9	BLD	BLD	0.51 ± 0.73
	E	BLD	2.3 ± 1.9	0.71 ± 0.94	BLD	BLD	BLD
Red algae	W	0.13 ± 0.01	0.09 ± 0.04	0.63 ± 0.89	BLD	0.12 ± 0.01	BLD
Nereid worm	W	0.80 ± 0.03	1.1 ± 0.1	1.2 ± 0.2	0.09 ± 0.00	0.14 ± 0.00	0.22 ± 0.03
	E	1.2 ± 0.1	0.75 ± 0.06	3.5 ± 0.1	0.11 ± 0.01	BLD	0.19 ± 0.00
Tube worm	W	1.2 ± 0.0	0.61 ± 0.06	1.3 ± 0.3	0.04 ± 0.02	BLD	0.15 ± 0.00
	E	0.14 ± 0.01	0.34 ± 0.02	0.91 ± 0.26	0.05 ± 0.01	0.05 ± 0.07	0.05 ± 0.06
Nerite snail	W	0.36 ± 0.02	0.75 ± 0.18	1.0 ± 0.6	0.06 ± 0.04	BLD	BLD
	E	0.55 ± 0.20	0.23 ± 0.05	0.53 ± 0.26	BLD	BLD	BLD
Drill shell	W	11 ± 2	5.1 ± 0.8	10.1 ± 2.2	0.74 ± 0.03	0.21 ± 0.00	2.5 ± 0.3
	E	0.60 ± 0.11	0.73 ± 0.00	2.2 ± 0.0	0.16 ± 0.16	BLD	0.08 ± 0.12
Rodong shell	W	0.28 ± 0.00	1.6 ± 0.6	4.8 ± 0.1	0.01 ± 0.02	BLD	BLD
	E	1.2 ± 0.0	4.7 ± 0.1	10.7 ± 2.8	0.17 ± 0.01	0.09 ± 0.12	0.22 ± 0.30
Green mussel	W	1.5 ± 0.1	1.1 ± 0.0	2.7 ± 1.1	0.05 ± 0.01	0.09 ± 0.13	2.4 ± 0.3
Lokan (clam)	W	3.8 ± 0.6	3.1 ± 0.7	4.5 ± 1.9	0.07 ± 0.00	0.22 ± 0.07	4.3 ± 2.7
	E	1.5 ± 0.1	1.0 ± 0.1	3.6 ± 0.8	0.12 ± 0.02	0.06 ± 0.08	0.12 ± 0.16

Leaf oyster	W	5.6 ± 0.9	1.8 ± 0.2	3.6 ± 0.8	0.12 ± 0.15	0.33 ± 0.16	2.2 ± 0.4
Mangrove oyster	E	4.5 ± 0.3	2.5 ± 0.6	8.5 ± 0.5	0.16 ± 0.01	0.21 ± 0.30	1.0 ± 0.1
Barnacles	W	3.1 ± 0.1	0.70 ± 0.05	4.4 ± 2.0	0.09 ± 0.03	BLD	0.67 ± 0.05
	E	3.2 ± 0.2	0.92 ± 0.07	4.8 ± 0.3	0.22 ± 0.04	0.10 ± 0.15	0.35 ± 0.49
Snapping prawn	W	0.10 ± 0.02	BLD	3.5 ± 0.7	BLD	BLD	BLD
	E	0.06 ± 0.00	BLD	2.8 ± 1.0	BLD	0.09 ± 0.12	BLD
Marine prawn	E	0.29 ± 0.10	0.57 ± 0.20	1.9 ± 0.7	0.31 ± 0.07	BLD	BLD
Tree climbing crab	W	0.18 ± 0.00	0.25 ± 0.02	0.59 ± 0.59	0.09 ± 0.00	0.05 ± 0.06	BLD
muscle	E	2.7 ± 0.0	1.0 ± 0.1	3.8 ± 1.0	0.11 ± 0.01	0.9 ± 0.13	0.19 ± 0.06
Tree climbing crab	W	0.33	1.3	5.3	0.27	0.50	BLD
egg	E	2.8	7.3	31	1.1	0.68	0.60
Thunder crab	W	4.1 ± 0.1	1.8 ± 0.1	4.1 ± 0.7	0.22 ± 0.01	0.24 ± 0.02	0.83 ± 0.11
	E	15 ± 4	5.8 ± 2.9	23 ± 11	2.0 ± 1.2	0.14 ± 0.06	2.1 ± 0.6
Half-beak	W	1.9 ± 0.2	2.3 ± 0.9	4.0 ± 0.9	0.30 ± 0.04	BLD	1.3 ± 0.5
	E	1.5 ± 0.5	2.0 ± 0.6	6.7 ± 2.0	0.35 ± 0.20	0.05 ± 0.06	1.6 ± 1.7
Mudskipper (muscle)	W	BLD	0.17 ± 0.03	0.22 ± 0.25	BLD	BLD	BLD
(eggs)		1.5	18	34	0.70	0.2	0.12
Goby (muscle)	E	1.2 ± 0.3	2.4 ± 2.1	24 ± 27	0.35 ± 0.01	BLD	BLD
(liver)	E	6.8	70	93	1.8	2.8	0.35
(eggs)	E	14	17	70	4.5	0.59	4.1

Glass perchlet	W	1.6 ± 0.3	1.5 ± 0.1	2.6 ± 1.3	0.07 ± 0.07	BLD	0.64 ± 0.40
Cardinalfish (muscle)	E	0.58 ± 0.10	1.6 ± 1.4	2.3 ± 1.3	0.18 ± 0.01	BLD	BLD
(liver)	E	45	40	190	9.9	1.9	11
Mullet (muscle)	W	18 ± 2	6.2 ± 0.4	6.7 ± 0.2	0.59 ± 0.07	0.26 ± 0.37	12 ± 8
(liver)	W	7.0	3.0	7.1	0.2	0.19	11
(muscle)	E	4.7 ± 0.2	4.0 ± 0.3	9.2 ± 1.1	0.42 ± 0.24	0.33 ± 0.04	1.0 ± 0.1
Archer fish (muscle)	W	2.5 ± 1.5	4.8 ± 3.0	5.1 ± 3.1	0.46 ± 0.35	0.13 ± 0.13	3.8 ± 2.7
(liver)	W	8.8	15	20	1.2	0.85	25
(muscle)	E	1.1	6.8	3.4	0.32	BLD	0.31
Chromide (muscle)	W	0.46 ± 0.16	1.7 ± 1.2	2.3 ± 2.3	0.23 ± 0.12	BLD	BLD
(liver)	W	14	47	90	4.8	0.73	4.2
(muscle)	E	1.5 ± 0.5	6.7 ± 4.3	7.6 ± 3.8	0.33 ± 0.22	0.18 ± 0.27	0.4 ± 0.3
(liver)	E	37	150	150	4.0	2.1	8.0

[a] W: Sungei Buloh, E: Sungei Khatib Bongsu.

PUBLICATIONS DERIVED FROM THIS WORK

Publications in scientific journals

Bayen S., Thomas, G.O., Lee, H.K. and Obbard J.P. 2003. Occurrence of PCBs and PBDEs in green mussels (*Perna viridis*) from Singapore, Southeast Asia. *Environ Toxicol Chem.* 10:2432-2437.

Bayen S., Gong Y., Chin H.S., Lee H.K., Leong Y.E. and Obbard J.P. 2004. Androgenic and Estrogenic Activity in Extracts from Green Mussel (*Perna viridis*) using a Human-Cell Based Bioassay *Environ Health Perspect* 112:1467-1471.

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Bayen S., Giusti P., Lee H.K. and Obbard J.P. Bioaccumulation of *p,p*'-DDT in Asian Seabass in a controlled aquaculture experiment. SETAC-ASE International Conference. 28-30 October 2003. Christchurch. New Zealand.

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Bayen S., Lee H.K. and Obbard J.P. Bioaccumulation of Persistent Organic Pollutants in Mangrove Ecosystems in Singapore.4th International conference on Marine Pollution and Ecotoxicology, 1-5 June 2004. Hong Kong.

Obbard J.P, Bayen S. and Lee H.K. Persistent organic pollutants in mangrove habitats in Singapore. 4th Asia Pacific Symposium on Environmental Geochemistry, 18-20 January 2005. Perth, Australia.

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